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(54) Title: SULFATED POLYSACCHARIDES AS INHIBITORS OF SMOOTH MUSCLE CELL PROLIFERATION (57) Abstract Highly sulfated oligosaccharides in the form of hexasaccharide and octasaccharide compounds which have antiproliferative activity with respect to smooth muscle cells are useful in treatment of conditions characterized by unwanted smooth muscle cell proliferation such as a result of trauma or disease states such as asthma, congestive heart failure and hypertension. The oligosaccharides have increased ability to inhibit the proliferation of smooth muscle cells and decreased ability to act as an anti-coagulant as compared with commercial heparin and/or unseparated fragments of heparin.		

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SULFATED POLYSACCHARIDES AS INHIBITORS OF
SMOOTH MUSCLE CELL PROLIFERATION

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Technical Field

The invention relates to the use of carbohydrate preparations as therapeutic and diagnostic compositions. In particular, the invention relates to polysaccharides having six or more saccharide units and compositions containing such polysaccharides which are useful in treating diseases and conditions characterized by excessive smooth muscle cell proliferation.

25

Abbreviations

In the representations of oligomers produced synthetically and those derived from heparin, the following abbreviations are used: D-glucuronic acid = GlcA; L-iduronic acid = IdoA; D-glucosamine = GlcNH₂; N-acetyl-D-glucosamine = GlcNAc; D-glucosamine N-sulfate = GlcNS; 2,5-anhydromannose = Man(2,5); 2,5-anhydromannitol = ManH(2,5); D-xylose = Xyl; glycosaminoglycan = GAG.

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The location of the O-linked sulfate residues is indicated by "S" and the number of the position of sulfation where the sulfate residue is linked to oxygen on the sugar residue. In these designations, also, the alpha and beta anomeric linkages are as those conventionally found in heparin and the indicated D or L configurations as conventionally found pertains. The locations of the sulfates are shown below the abbreviation for the sugar to which they apply, thus, for example,

IdoA-GlcNS

2S 6S

refer to L-iduronic acid and D-glucosamine N-sulfate with sulfates connected respectively at the 2 and 6 positions of the sugar residues.

Background Art

Proliferation of smooth muscle cells in blood vessel walls occurs in response to vascular injury, and in association with certain disease states (Austin, G.E., et al., J Am Coll Cardiol (1985) 6:369-375). The proliferation of these cells can have negative effects due to the production of excess proteins or other matrix molecules, which, along with the cells themselves, form pathologic lesions of, for example, atherosclerosis, renal hypertension, pulmonary hypertension, vasculitis, and post-surgical vascular retinosis. These results are distinguished from the acute response to trauma characterized by blood clotting.

Glycosaminoglycans (GAG) are copolymers of alternating hexosamine and aldouronic acid residues which are found in sulfated forms and are synthesized as proteoglycans. They have collectively been called muco-

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polysaccharides, and those in heparin are more precisely called glycosaminoglycuronans.

To place the compositions discussed below in context, it may be noted that heparin and heparan sulfate are members of the GAG family which are classified by the nature of the hexosamine/aldouronic acid repeating units. For example, in chondroitin sulfates, the aldouronic acid is primarily D-glucuronic acid, and the hexosamine is N-acetylated 2-amino-2-deoxy-D-galactose, more commonly known as N-acetyl galactosamine and abbreviated as GalNAc.

In dermatan sulfate (chondroitin sulfate B) the aldouronic acid is mostly L-iduronic acid and the hexosamine is GalNAc. In keratan sulfate, the aldouronic acid is replaced by D-galactose, and the hexosamine is mostly N-acetylated 2-amino-2-deoxy-D-glucose, more commonly known as N-acetyl glucosamine and abbreviated as GlcNAc.

In the compositions of interest herein, heparan sulfate and heparin, the hexosamine is mostly N-acetylated or N-sulfated glucosamine (GlcN), and the aldouronic acid is mostly L-iduronic in heparin and mostly D-glucuronic acid in heparan sulfate. Heparan sulfate is commonly considered to have a higher proportion of glucuronic acid than heparin.

Problems of heterogeneity in preparations of heparan sulfate or heparin isolated from tissues make sharp distinctions difficult, since these oligosaccharides are related by the biosynthesis pathway, as explained below. Conventional heparin (used as an anticoagulant) has a molecular weight of 5-25 kDa and is extracted as a mixture of various chain lengths by conventional procedures. These procedures involve autolysis and extraction of suitable tissues, such as

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beef or porcine lung, intestine, or liver, and removal of other GAGs as well as nonpolysaccharide components.

5 The molecular weight of the chains in the extract is significantly lower than the 60-100 kd known to exist in the polysaccharide chains of the heparin proteoglycan synthesized in the tissue. The GAG moiety is synthesized bound to a peptide matrix at a serine residue through a tetrasaccharide linkage region of the sequence D-GlcA-D-Gal-D-Gal-D-Xyl → protein, which is
10 then elongated at the D-GlcA residue with alternate additions of GlcNAc and GlcA.

The polysaccharide sidechains are modified by a series of enzymes which sequentially deacetylate the N-acetyl glucosamine and replace the acetyl group with
15 sulfate, epimerize the hydroxyl at C5 of the D-glucuronic acid residue (to convert it to L-iduronic acid), sulfate the O-2 of the resulting L-iduronic acid and the O-6 of the glucosamine residue. Some of the chains are further sulfated at the O-3 of the glucosamine residue, either at
20 the heparan or heparin stage. This latter sulfation generates the active sequence required for anti-thrombin III binding and thus anticoagulation activity. Other chemically possible sulfation sites are on the O-2 of D-glucuronic acid.

25 Due to their obvious chemical similarity, isolated "heparin" may contain considerable amounts of what might otherwise be classified as heparan sulfate.

There is an extensive body of art concerning depolymerization of heparin/heparan sulfate chains and
30 separation of products by size. Particularly relevant is the report of Guo, Y. et al., Anal Biochem (1988) 168:54-62 which discloses the results of structure determination after the 2,5-anhydromannose at the

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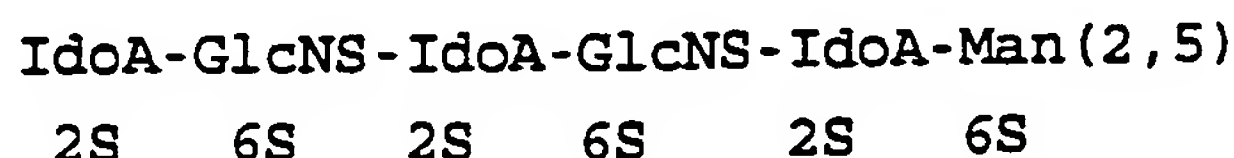
reducing terminus is reduced to the corresponding 2,5-anhydromannitol.

The involvement of heparin or heparan sulfate or degradation products thereof in smooth muscle proliferation has been recognized for some time. Heparin and heparan sulfate can slow or arrest the vascular smooth muscle cell proliferation associated with injury described hereinabove (Clowes, A.W., et al., Nature (1977) 265:625-626). The effect of heparan sulfate and heparin on smooth muscle cell proliferation is also described by Marcum, J.A., et al. in Biology of Proteoglycan, Academic Press (1987) pp. 301-343. The inhibition of vascular smooth muscle cell growth by heparin was further described by Castellot, J.J., Jr., et al., J Biol Chem (1982) 257:11256-11260 and the effect of heparin on vascular smooth muscle cell growth in fetal tissue was described by Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7. The effect of heparin as an inhibitor of both pericyte and smooth muscle cell proliferation was shown by Orlidge, A., et al., Microvascular Research (1986) 31:41-53, and these authors further showed that chondroitin sulfate and dermatan sulfate do not have this effect. A review of the effects of heparin and heparan sulfate on the proliferation of smooth muscle cells is by Benitz, W.E. in "The Pulmonary Circulation: Normal and Abnormal", Fishman, A.P., ed., University of Pennsylvania Press (1988).

It is not clear by what mechanism these glycosaminoglycans operate, or to what extent they interact with other growth factors such as epithelial and fibroblast growth factors. It has been proposed that a 3-O sulfate on glucosamine in an oligosaccharide of at least 5 sugars is important in this process and that both O- and N-sulfation is important (Castellot, J.J., et al., J

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Cell Physiol (1984) 120:315-320; Castellot, J.J., et al.,
J Cell Biol (1986) 102:1979-1984). Hexasaccharides-
decasaccharides obtained from partial nitrous acid diges-
tion of heparin bind to acidic fibroblast growth factor
and aid its mitogenic activity in fibroblasts, but
inhibit the proliferation of endothelial cells under some
conditions (Barzu, T., et al., J Cell Physiol (1989)
140:538-548). The effective hexasaccharide was stated to
have the structure:



Others have indicated that the presence of 2-
O-sulfate glucuronic acid is not necessary for antipro-
liferative activity (Wright, Jr., T.C., et al., J Biol
Chem (1989) 264:1534-1542). In this article, size
separated fragments of defined length prepared by nitrous
acid cleavage and gel filtration were further separated
according to charge for some assays. Partially digested
heparin separated only according to size was tested with
respect to stimulation of the growth of smooth muscle
cells and epithelial cells. Similar results were found
in both cases, although the results were not identical.
Tetrasaccharides of the type tested were shown to have
very low antiproliferative activity; hexasaccharides,
octasaccharides, and decasaccharides were shown to be
active to approximately the same level on a weight/volume
concentration basis. Also tested was a synthetic
pentapeptide which represents a unique sequence of the
heparin required for the binding of heparin to
antithrombin III; this pentapeptide was active in
inhibiting proliferation for smooth muscle but not
epithelial cells. The size-separated fractions were

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then treated chemically to produce "O-oversulfation" and this treatment enhanced the inhibitory activity; indeed, oversulfation of the tetrasaccharide fragment preparation yielded a tetrasaccharide fraction which was active
5 in inhibiting proliferation. The converse process, comprising desulfation and reacetylation of the amino groups of glucosamine results in a reduction in antiproliferative activity. These fragments could, however, be made more active by subsequent oversulfation.

10 Also capable of reducing the activity of the heparin fragments was reduction of the carboxyl groups so as to reduce the total negative charge. O-oversulfation partially restores this activity. These results with N-desulfated, N-acetylated fragments which are lacking in
15 antiproliferative activity is distinguishable from previous results wherein similarly treated heparin retains the capacity to prevent cell division because of the size dependency of the antiproliferative activity--larger fragments being more powerful in general than
20 smaller ones.

When the size separated fraction was further fractionated according to charge, it was found that the most highly charged fractions showed the greatest activity. Furthermore, it was shown that although the
25 synthetic pentasaccharide identified as the antithrombin III binding site is capable of inhibiting proliferation in smooth muscle cells, any treatment of heparin which would destroy the sequence corresponding to this pentapeptide (i.e., periodate treatment) does not destroy
30 antiproliferative activity.

Methods of synthesizing oligosaccharides are disclosed in U.S. Patent 4,943,630 issued July 14, 1990 which is incorporated herein by reference to disclose such methods.

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The present inventors have now found that an enhanced antiproliferative activity with respect to smooth muscle cells is associated with an oligosaccharide portion of the heparin or heparan sulfate GAGs which is highly sulfated and contains 6 or 8 saccharide units and have provided synthesis mechanisms for making polysaccharides containing 6 or more sugar residues, which oligosaccharides have enhanced antiproliferative activity with respect to smooth muscle cells.

10

Disclosure of the Invention

The invention provides a low molecular weight glycosaminoglycan (GAG) composition which has superior specific antiproliferative activity with regard to smooth muscle cells. The existence of this activity in a low molecular weight GAG provides the opportunity for effective pharmaceutical compositions which can be prepared by synthesis or by isolation of the composition from natural sources.

20

Accordingly, in one aspect, the invention is directed to a process to prepare a sulfated polysaccharide having antiproliferative activity. The polysaccharides of the invention may be produced synthetically using a sequence of chemical reactions as disclosed herein or obtained by digesting heparin and carrying out separation procedures based on size and charge as disclosed herein.

25

In order to produce the polysaccharide compounds of the invention synthetically it is first necessary to synthesize an iduronic acid synthon. Next, a glucosamine synthon is produced. The iduronic acid synthon and glucosamine synthon are reacted to produce a disaccharide synthon. The disaccharide units can be reacted to form oligosaccharides containing 4, 6, 8 or

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any multiple thereof of monosaccharide units and/or can be reacted with either an iduronic or glucosamine reaction synthon to provide oligosaccharides containing any odd number of saccharide units.

5 In order to obtain the oligosaccharide compounds by digestion, the heparin is obtained from a natural source and subjected to digestion with nitrous acid under conditions which favor the formation of an oligosaccharide mixture containing large amounts of hexa-
10 and octasaccharides. Following the digestion, the mixture is separated according to size and those factions corresponding to hexa- and octasaccharides are combined and recovered. The recovered portions are then separated according to charge in order to obtain the more highly
15 charged fractions. These fractions will contain oligosaccharides which are highly sulfated. Polysaccharides sulfated at the 0-3 position of the GlcN (associated with anticlotting activity) are not encompassed by the present invention.

20 The invention is also directed to pharmaceutical compositions comprised of the oligosaccharides of the invention either alone or in combination with excipients, i.e., pharmaceutically acceptable materials with no pharmacological effect.
25 Such compositions may be administered to a patient in order to regulate smooth muscle cell proliferation.

 A primary object of the present invention is to provide synthetically produced oligosaccharides containing 6 or more monosaccharide units, which are
30 highly sulfated at particular positions other than 0-3 positions of the GlcNs and which effect smooth muscle cell proliferation.

 Another important object of the present invention is to provide a method of obtaining hexa- and
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octasaccharide units from natural heparin and heparan sulfate which hexa- and octasaccharide units are effective in regulating smooth muscle cell proliferation and which do not process any significant degree of anticlotting activity.

5 An advantage of the present invention is that the oligosaccharide units can be formulated into pharmaceutical compositions which can be administered to aid in the regulation of smooth muscle cell proliferation.

10 A feature of the present invention is that the oligosaccharide units include monosaccharide residues which are sulfated at particular positions (other than the 0-3 position) which effected the ability of the oligosaccharide to regulate smooth muscle cell proliferation.

15 These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure, synthesis and usage as more fully set forth below, reference being made to the accompanying figures and general structural formulas forming a part herein wherein like symbols refer to like molecular moieties throughout.

25

Brief Description of the Drawings

Figures 1A and 1B show the elution profiles from gel filtration chromatography of reaction mixtures produced using varying amounts of nitrous acid.

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Figure 2 shows the growth inhibition activity of the various sized fractions.

Figures 3A and 3B show the elution profiles of hexasaccharide and octasaccharide subunits, respectively, from DEAE-Toyopearl chromatography.

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Figures 4A and 4B show the growth inhibition activity of various fractions collected in the elution profiles of Figures 3A and 3B.

Figure 5A shows the elution profile from
5 reverse-phase ion-pairing HPLC for the S-6 fraction shown in Figure 3A.

Figure 5B shows a comparable profile for the total hexasaccharide fraction.

Figures 6A, 6B and 6C are charts showing pos-
10 sible sulfated positions for fifty-seven octasaccharides of the invention.

Detailed Description of Preferred Embodiments

Before the present oligosaccharides and
15 processes for making and formulating such are described, it is to be understood that this invention is not limited to the particular oligosaccharides, formulations or processes described as such compounds, compositions and methods may, of course, vary. It is also to be
20 understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

25 It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an oligosaccharide" includes mixtures of
30 oligosaccharides and, reference to "an octasaccharide" includes reference to mixtures of octasaccharides of the type described herein and reference to "the process step" or "the process" includes reference to various steps and processes of the type described herein which will be
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known to those skilled in the art or which will become apparent to those skilled in the art upon reading this disclosure and so forth.

5 Definitions

By "heparin/heparan sulfate" or "heparin" is meant a preparation obtained from tissues in a manner conventional for the preparation of heparin as an anti-coagulant or otherwise synthesized and corresponding to that obtained from tissue. See Conrad, H.E., Heparin and Related Polysaccharides, Vol. 56, p. 18 of Annals of N.Y., Academy of Sc., June 7, 1989, incorporated herein by reference. This preparation may include residues of D-glucuronic acid (GlcA), as characteristic of heparan sulfate as well as iduronic acid (IdoA) as characteristic of heparin. However, both GlcA and IdoA are present in both, they are present in different proportional amounts. The (IdoA)/GlcA ratio increases as heparan sulfate becomes more heparin-like. As described in the Background section above, the conversion of D-glucuronic acid to L-iduronic acid is a result of epimerization at the 5 carbon of GlcA residues in a heparan-type intermediate. This sequence of steps involved in such epimerization and conversion is understood in the art. To the extent that full conversion has not been made, heparan sulfate characteristics remain in the preparation. Because the precise nature of the polymeric chains in the preparations of heparin is not generally determined, and varies from preparation to preparation, the term "heparin/heparan sulfate" or "heparin" is intended to cover the range of mixtures encountered. Perhaps the main feature which distinguishes heparan sulfate from heparin is that the latter has anti-coagulant activity.

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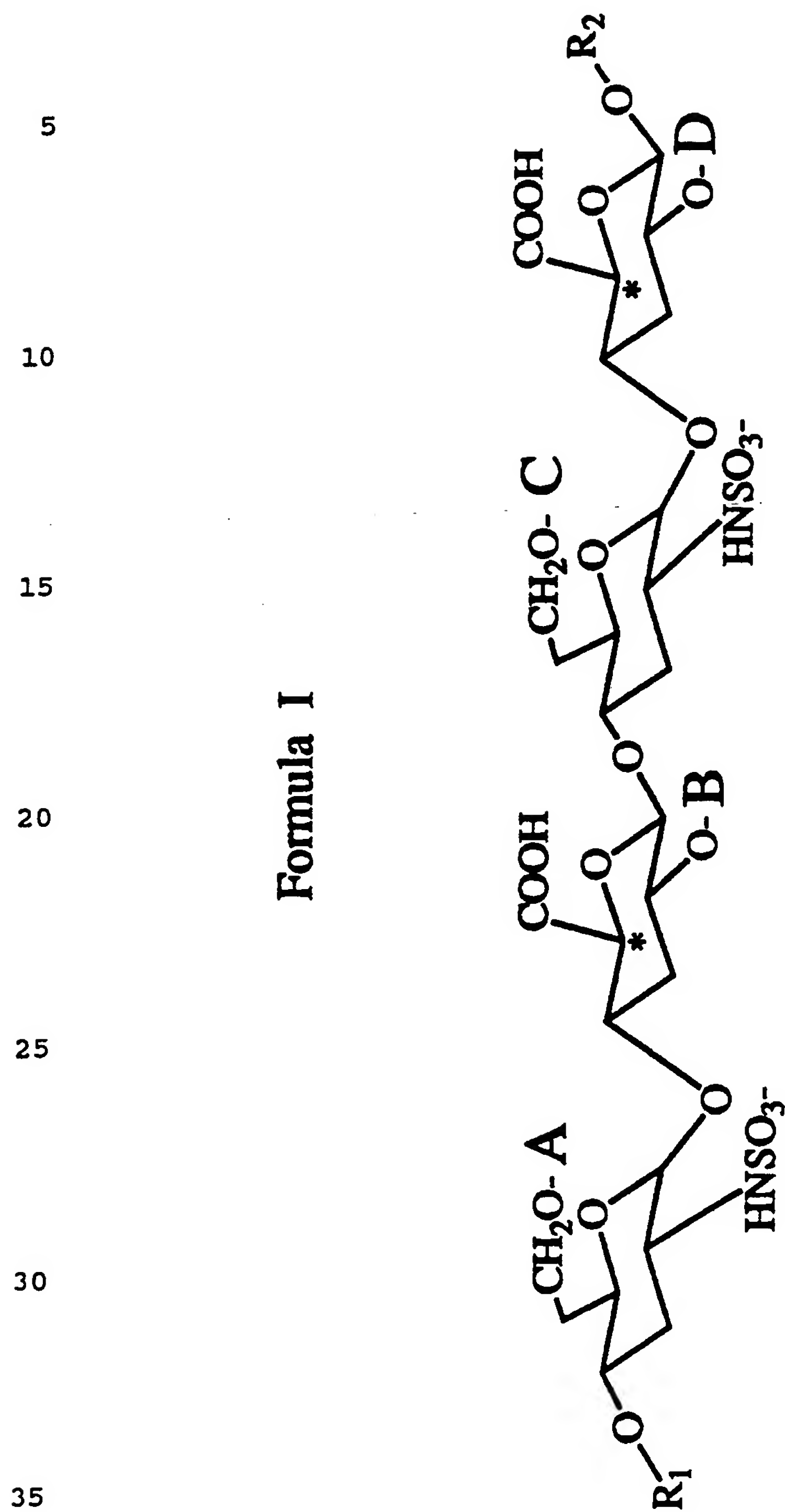
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The "heparin/heparan sulfate" preparation can be obtained from a variety of mammalian tissues, including, if desired, human tissue. Generally, porcine or bovine sources are used, and vascularized tissues are preferred. A preferred source of heparin/heparan sulfate starting material is porcine intestinal mucosa, and preparations labeled "heparin" prepared from this tissue source are commercially available. In general, the heparin/heparan sulfate starting material is prepared from the selected tissue source by allowing the tissue to undergo autolysis and extracting the tissue with alkali, followed by coagulation of the protein, and then precipitation of the heparin-protein complex from the supernatant by acidification. The complex is recovered by reprecipitation with a polar nonaqueous solvent, such as ethanol or acetone or their mixtures, and the fats are removed by extraction with an organic solvent such as ethanol and proteins by treatment with a proteolytic enzyme, such as trypsin. Suitable procedures for the preparation of the heparin starting material are found, for example, in Charles, A.F., et al., Biochem J (1936) 30:1927-1933, and modifications of this basic procedure are also known, such as those disclosed by Coyne, E., in Chemistry and Biology of Heparin, Elsevier Publishers, North Holland, New York, Lunblad, R.L., et al., eds. (1981).

Synthetic Oligosaccharides

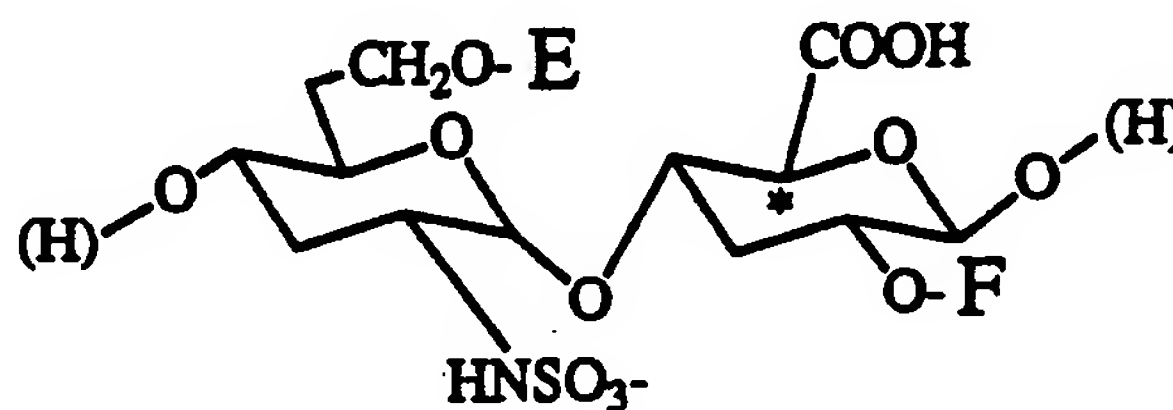
The synthetic oligosaccharides of the present invention include at least 6 saccharide residue units and have the following general structural formula:

Formula I



(The hydroxyl groups on the 3-position of the sugars have been omitted for greater clarity) and the * adjacent the carbon substituted with COOH indicates (here and throughout the application) undetermined stereochemistry which may be any possible stereochemistry for the molecule; wherein each of the variables A, B, C and D are independently hydrogen or SO_3R with the proviso that at least 2 of the variables are SO_3R and each R is independently H^+ , Na^+ , or other suitable cation; and wherein R_1 and R_2 are each independently hydrogen, or one or more repeating units having the following structure:

Formula I(a)



wherein when the unit of structural Formula I(a) is connected at one end the hydrogen is not present and the hydrogen at the end not connected is present and further wherein each of the variables E and F is independently hydrogen or SO_3R .

Some preferred embodiments of the present invention include compounds of structural Formula I wherein each of A, B, C and D are $-\text{SO}_3^-$ and either R_1 or R_2 is the unit of structural Formula I(a). Another preferred embodiment includes compounds of structural Formula I wherein each of A, B, C and D is $-\text{SO}_3^-$ and both

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R_1 and R_2 are the unit of structural Formula I(a) and wherein each E and F of the unit I(a) is $-\text{SO}_3^-$.

Polysaccharides Derived from Heparin and/or Heparan

5 Sulfate

Preferably, the heparin/heparan sulfate preparation used as a starting material is first purified by extraction with a solvent in which the heparin is insoluble, such as ethanol or acetone. The purified starting material is then depolymerized.

Depolymerization in general can use various reagents, such as nitrous acid, heparinase or periodate. The antiproliferative compositions of the invention are obtainable when partial nitrous acid digestion is conducted under conditions which maximize formation of hexasaccharide and octasaccharide fragments.

In typical procedures, the nitrous acid is prepared in situ by acidification of a solution of sodium nitrite at a concentration of 50 mM, and the reagent is used to treat the heparin at a concentration of about 60-180 mg/ml, at a pH of about 1.0 to about 2.0, preferably about 1.5. The reaction is conducted at room temperature and can be neutralized by addition of a suitable reagent at the desired stage of digestion. Other depolymerization methods can be used as long as they produce active components, i.e., components which (1) are predominantly hexa- and octasaccharides; (2) are heavily sulfated; (3) have substantial antiproliferation activity with respect to smooth muscle cells; and (4) have insignificant or no anticlotting activity.

Isolated fragments can then be tested for their ability to inhibit smooth muscle cell proliferation. Fragments with high activity with respect to inhibiting the proliferation of smooth muscle cells and low activity

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with respect to their ability to inhibit blood coagulation (relative to commercial heparin) are preferred.

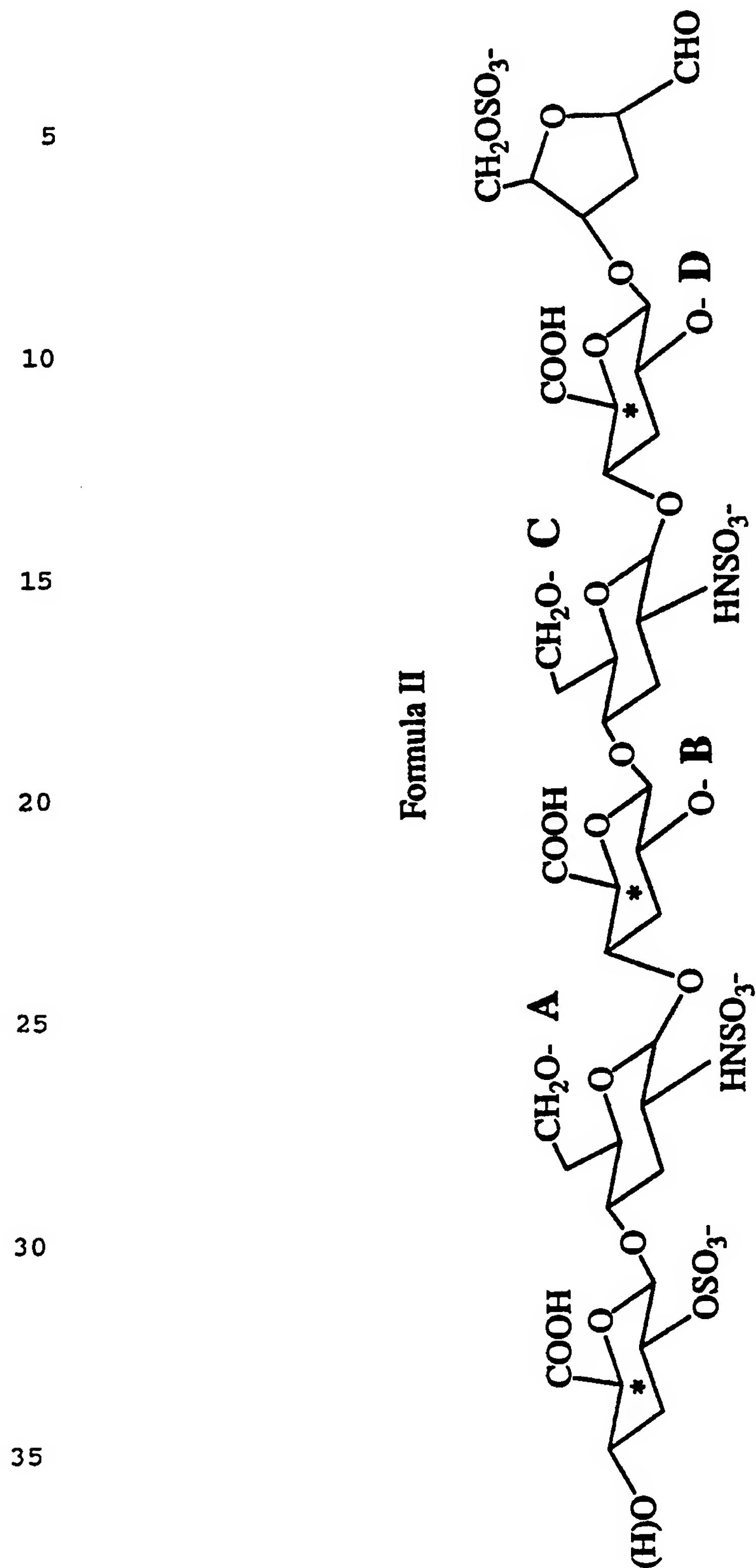
The depolymerization results in a mixture of fragments that is then separated on the basis of size. A variety of size separation techniques are available, including gel permeation, density gradient centrifugation; especially preferred is gel filtration chromatography using a Sephadex or polyacrylamide gel system with a fractionation range of about 100-3500 daltons. A particularly preferred gel permeation resin is Biogel P10, and upon separation using this method, fragments which are disaccharides, tetrasaccharides, hexasaccharides, octasaccharides, and oligosaccharides of higher molecular weights are effectively separated.

The fractions containing predominantly hexa- and octasaccharide units show enhanced activity in inhibiting the proliferation of smooth muscle cells. Verification of this property can be obtained using standard assays, such as those described in Castellot, J.J. Jr., et al., J Cell Biol (1986) 102:1979-1984. Other assay methods, such as those of Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7 can also be used.

The hexasaccharide fragments thus obtained are of the formula:

30

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wherein each of the variables A, B, C and D is independently H or SO_3R and each R is independently H or a cation, with the proviso that at least two of the variables A, B, C or D is $-\text{SO}_3\text{R}$. It is pointed out that
5 hydroxyl groups on 3-positions of the sugars have been omitted for greater clarity and the * adjacent the COOH indicates undetermined stereochemistry.

In the compounds of formula (II) the sugar at the reducing terminus is deaminated to form the
10 2,5-anhydromannose shown. When this compound is further reduced, the CHO shown becomes $-\text{CH}_2\text{OH}$; however, this reduction does not occur in the depolymerization reaction per se. This reduced form is a compound of the present invention as shown below in formula (IIa).

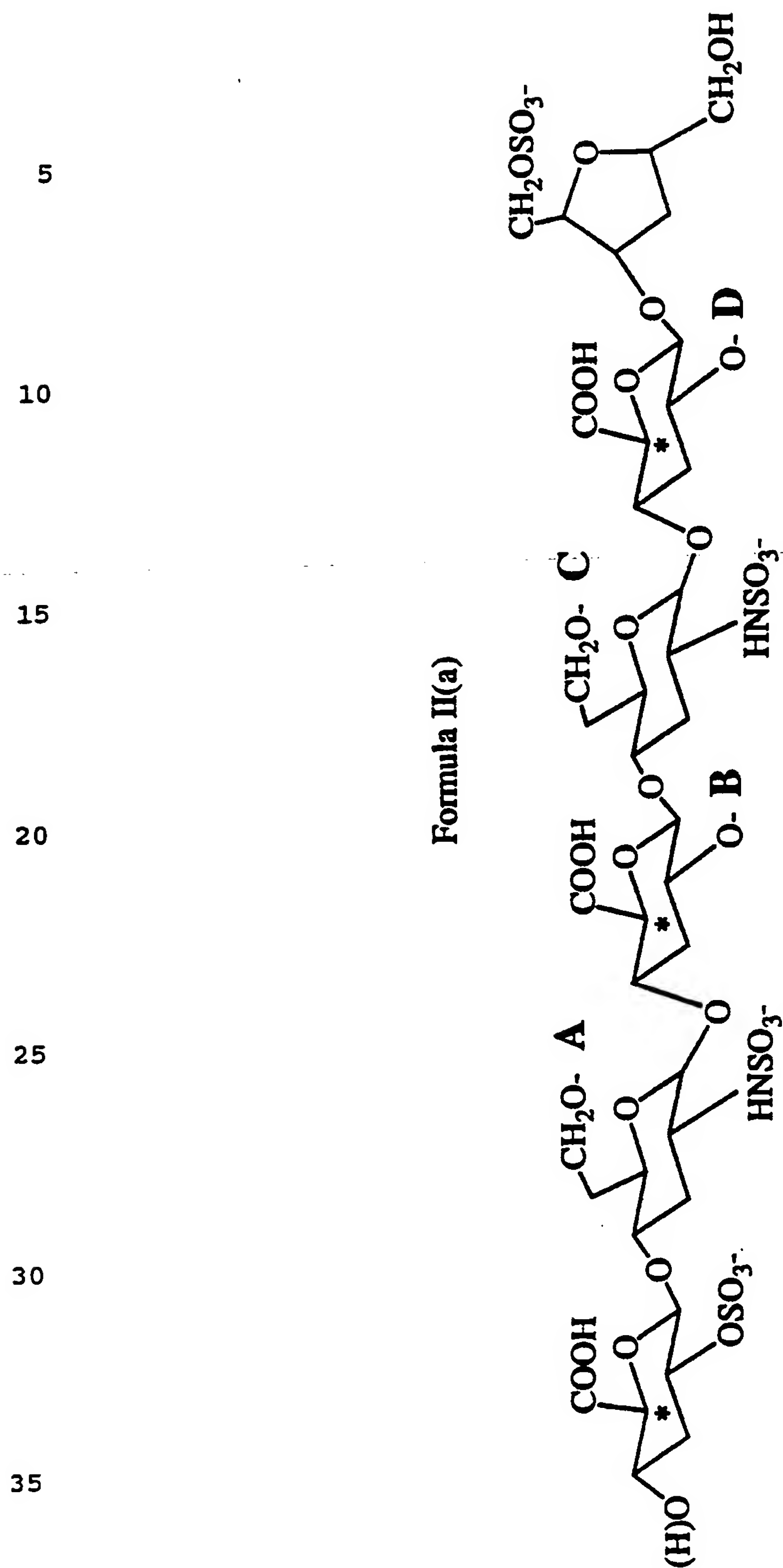
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In the $-\text{SO}_3\text{R}$, the cations represented by R can either be inorganic cations such as sodium, potassium, calcium, or ammonium ion or can be organic cations such as those obtained from quaternary amines; these salts are
5 formed by simple neutralization.

Based on the above formula (II) it can be seen that there are eleven different possible configurations with respect to the positions of $-\text{SO}_3\text{R}$ moieties when 2 or more are present. These configurations are schematically
10 shown in the following table wherein an "X" indicates a $-\text{SO}_3\text{R}$ is present at the indicated A, B, C or D position.

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Table 1

		A	B	C	D	= SO3
5	1.	X	X	X	X	
	2.	X	X	X		
	3.		X	X	X	
	4.	X		X	X	
	5.	X	X		X	
10	6.	X	X			
	7.	X		X		
	8.	X			X	
	9.		X	X		
	10.		X		X	
15	11.			X	X	

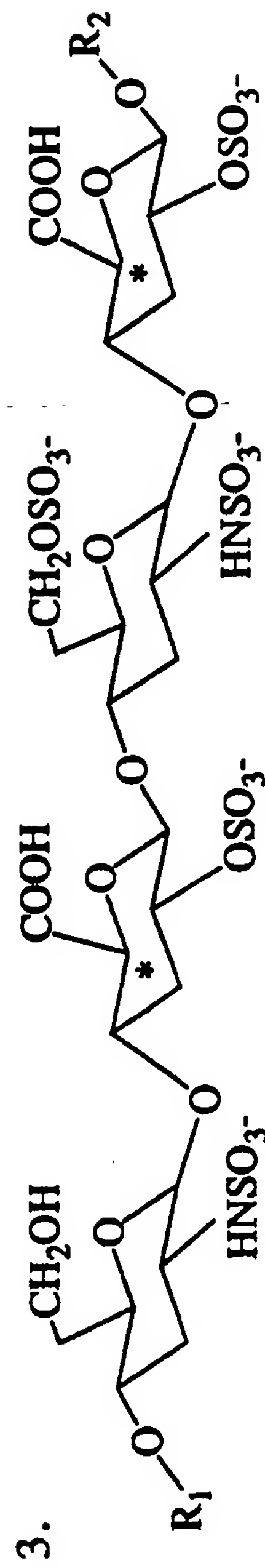
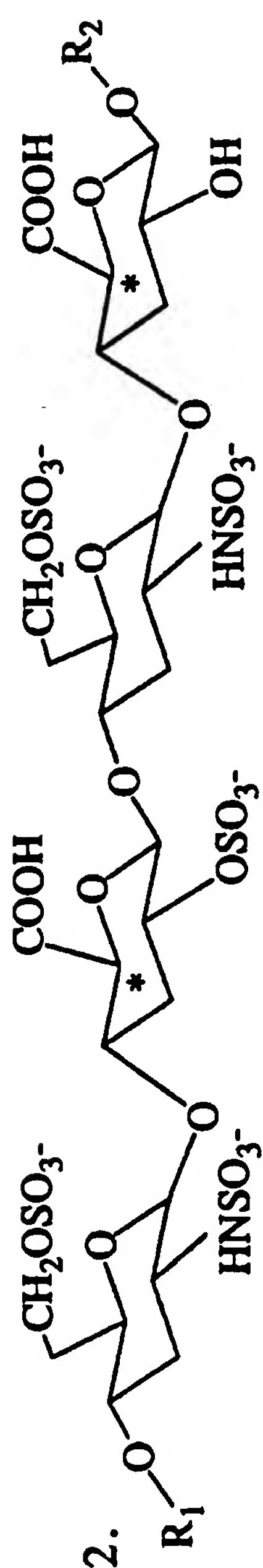
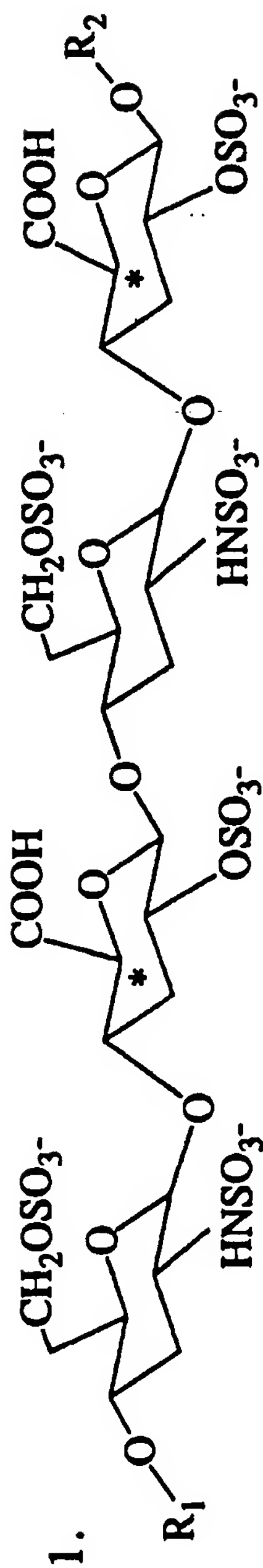
In that each "R" can be any cation, the above eleven possible structures represent a significantly larger number of compounds, i.e., the acid and salt forms.

20 The basic structure of the eleven possible configurations shown by formula (1) and the above table are put forth below. Regarding the structures II(1) - II(11) it is pointed out that hydroxyl groups on the 3-position of the sugars have been omitted for greater
25 clarity.

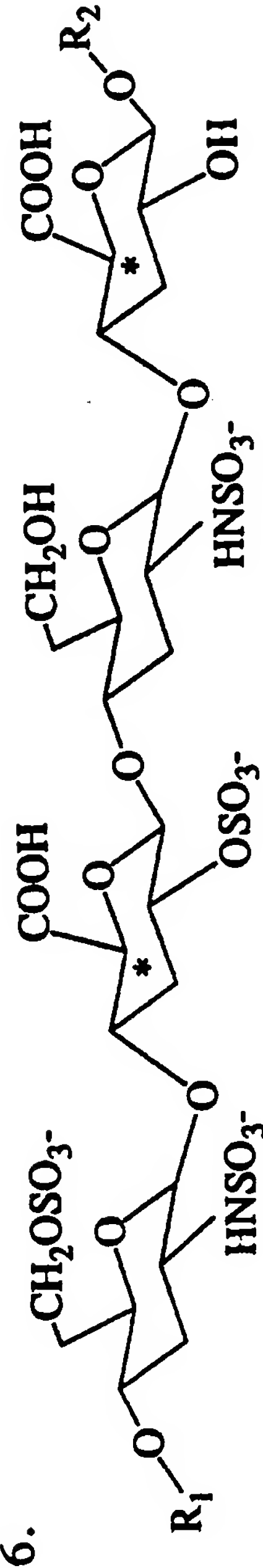
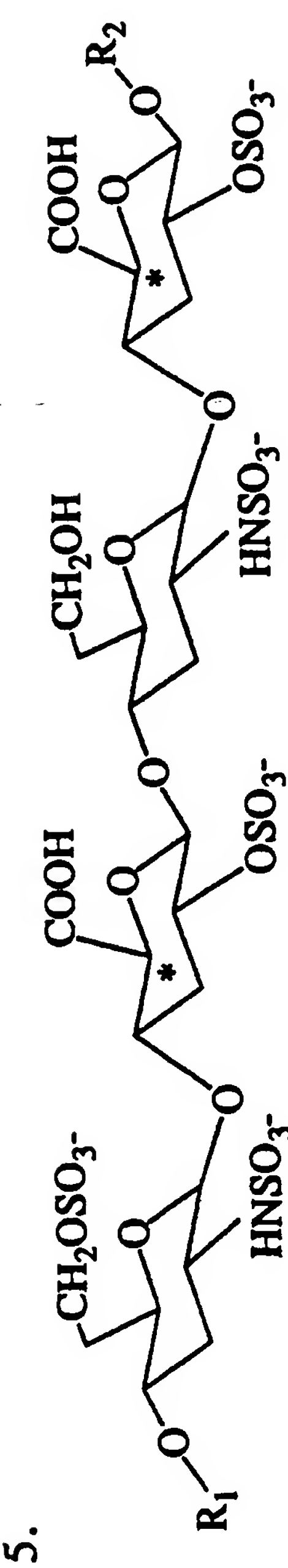
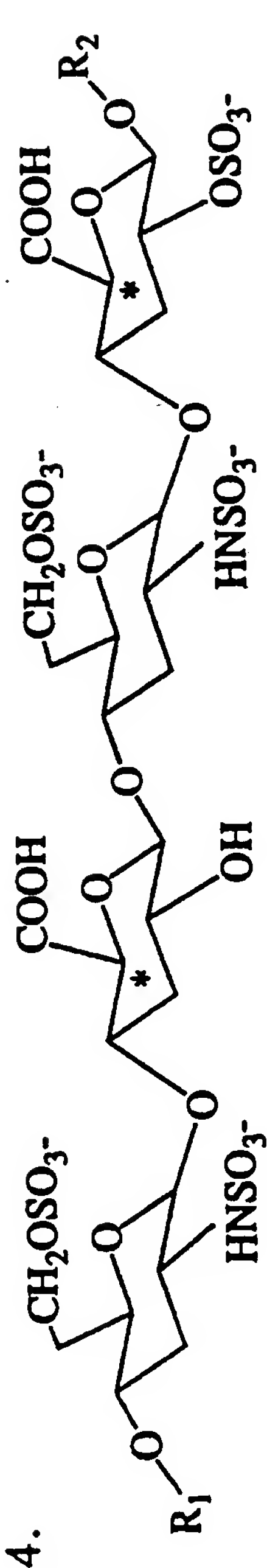
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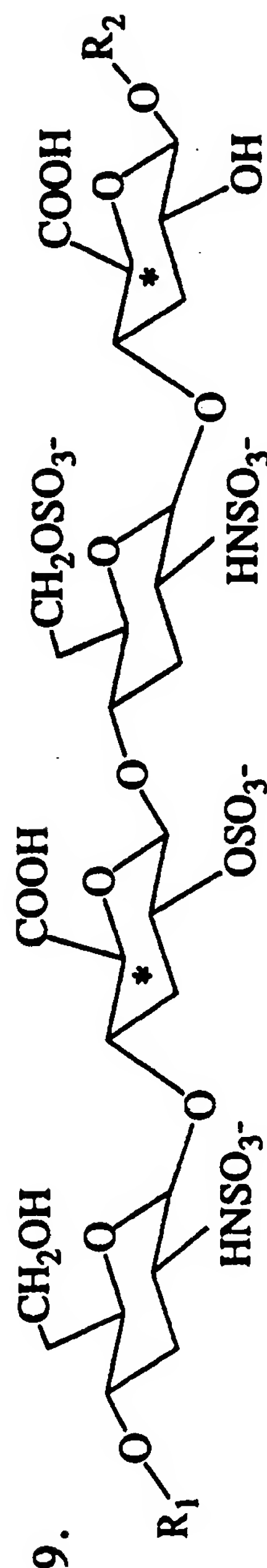
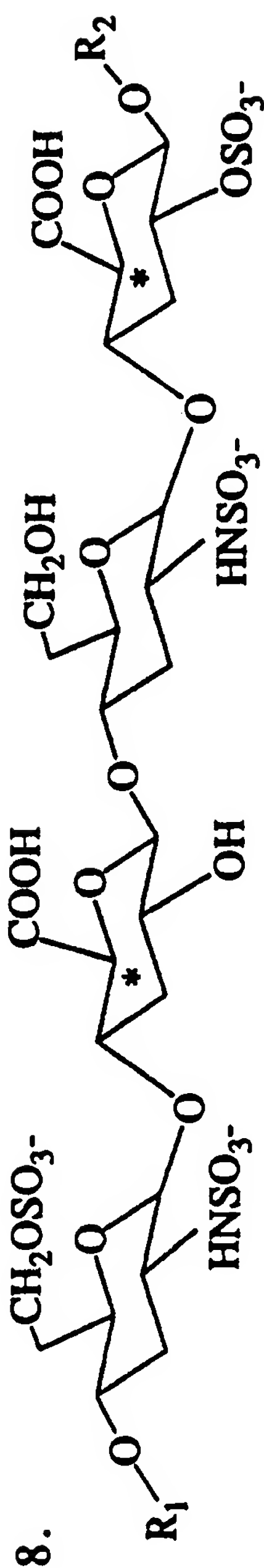
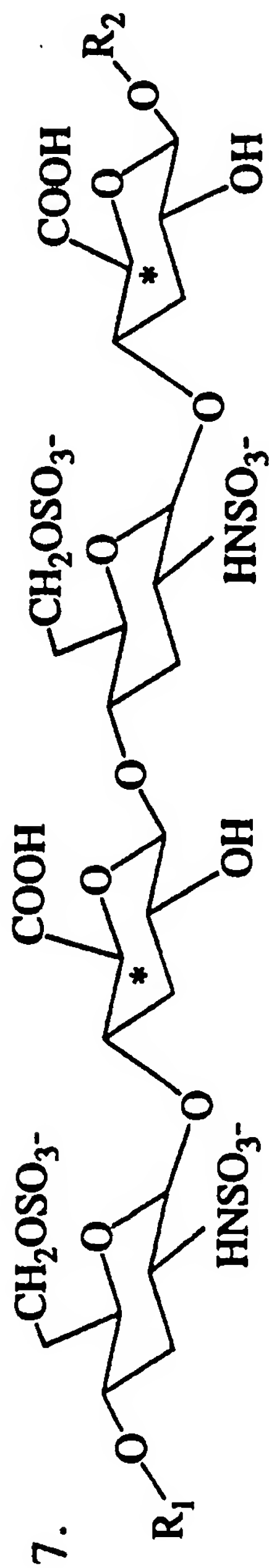
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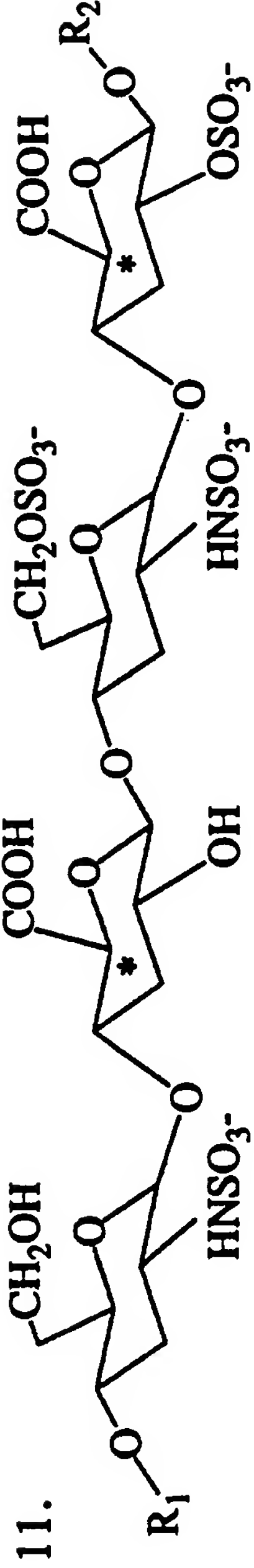
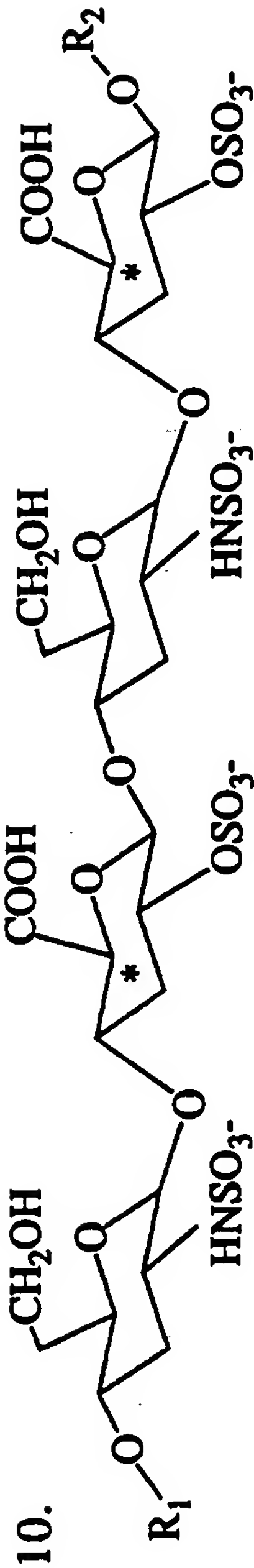
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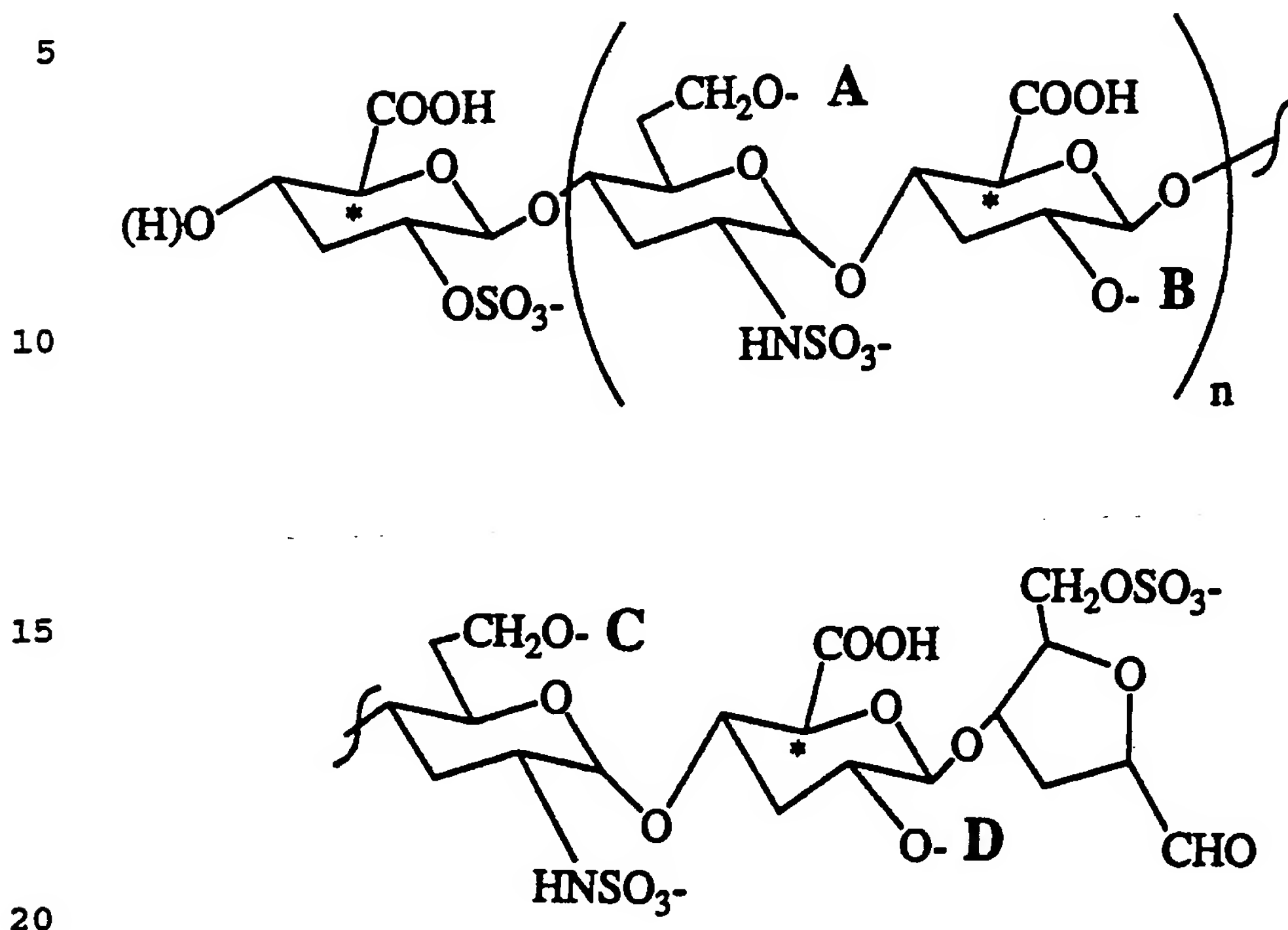
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Representative compounds of the invention, wherein R is as above defined are set forth as follows. In these representations, the following abbreviations are used: L-iduronic acid = IdoA; D-glucosamine = GlcNH₂; N-acetyl-D-glucosamine = GlcNAc; D-glucosamine N-sulfate = GlcNS; 2,5-anhydromannose = Man(2,5); 2,5-anhydromannitol = ManH(2,5). The location of the O-linked sulfate residues is indicated by "S" and the number of the position of sulfation where the SO₃R residue is linked to oxygen. In the designations below, the alpha and beta anomeric linkages are as those shown in formula 1 above and the indicated D or L configurations as set forth above pertains. The locations of the sulfates are shown below the abbreviation for the sugar to which they apply.

The hexasaccharide and octasaccharide fragments obtained by digesting heparin and following the above-described procedures are of the formula:

Formula III



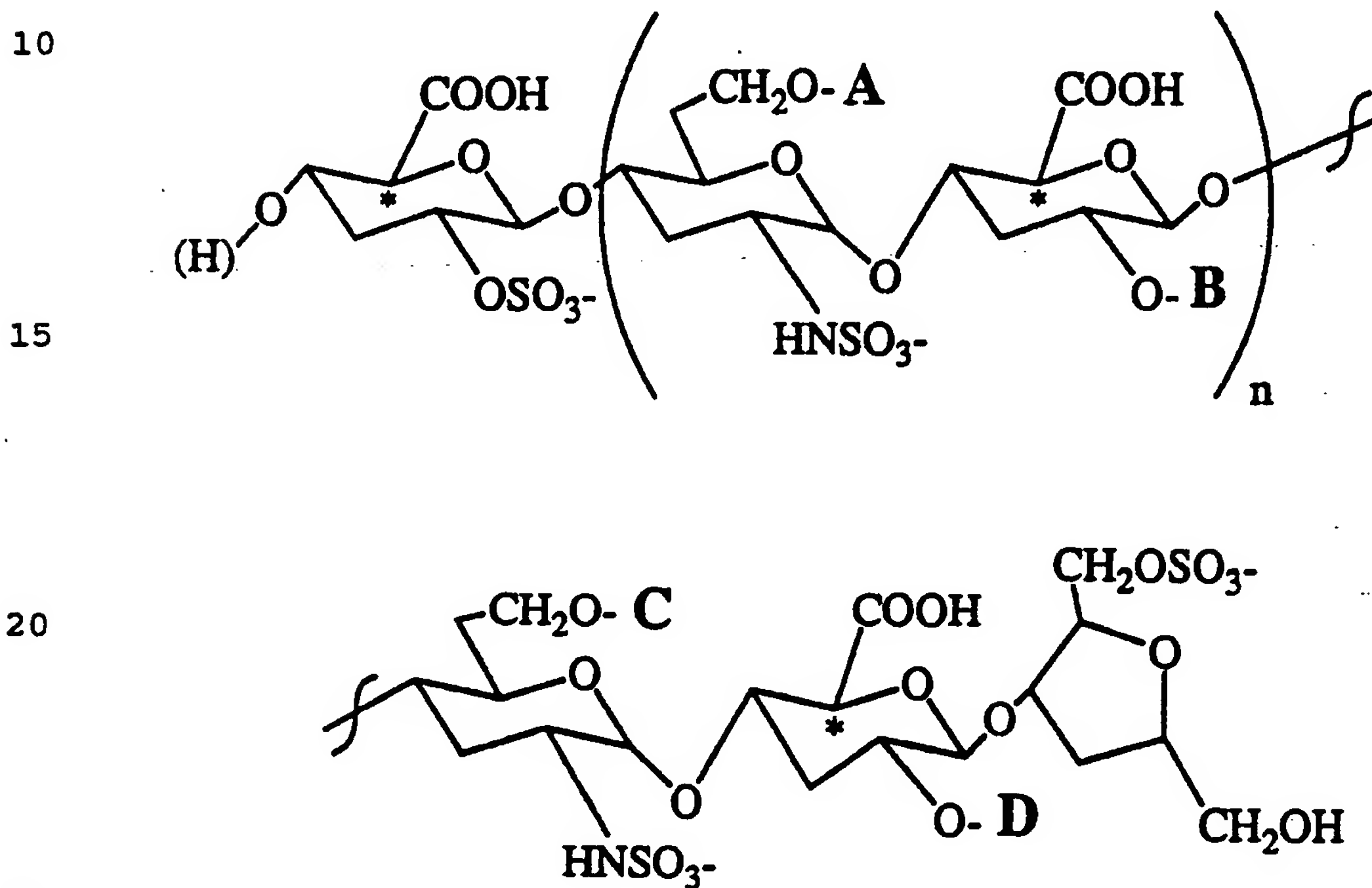
wherein n is 1 or 2, each of the variables A, B, C and D is independently H or SO_3R , wherein each R is independently H or a cation, with the proviso that at least two of said A, B, C and D are SO_3R . As in the Formulas I and II above, the hydroxyl groups in the 3 position of the sugars have been omitted for greater clarity and the asterisk next to the position of the carboxyl group indicates that the stereochemistry thereof is undetermined.

In the compounds of formula (III) the sugar at the reducing terminus is deaminated to form the 2,5-anhydromannose shown. When this sugar is further reduced, the CHO shown becomes $-\text{CH}_2\text{OH}$; however, the

reduction does not occur in the depolymerization reaction per se. The reduced compound is part of the present invention and is shown below as formula (IIIa) wherein each of the variables is defined as in formula III above.

5

Formula III(a)



25

The cations represented by R can either be inorganic cations such as sodium, potassium, calcium, or ammonium ion or can be organic cations such as those obtained from quaternary amines and these salts are formed by simple neutralization. As above, the hydroxyls at the 3 positions are not shown in the structure, but are understood to be present, and the asterisk adjacent the position of the carboxyl groups indicates that the stereochemistry at these positions is undetermined.

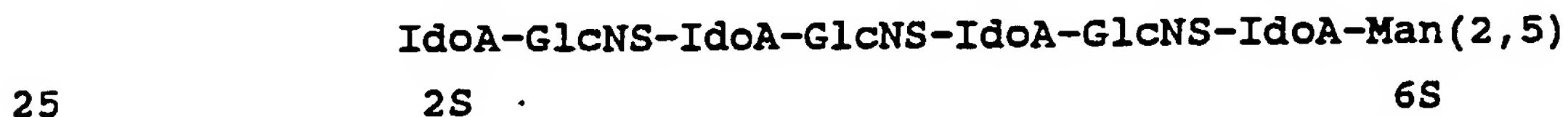
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Based on the above formula (III) it can be seen that there are fifty-seven different possible configurations with respect to the position of the $-SO_3R$ moieties when 2 or more are present. These configurations are schematically shown in Figures 6A, 6B and 6C wherein an "X" indicates a $-SO_3R$ is present at the indicated A, B, A', B', C or D position, wherein A' and B' represent the embodiments of A and B in the parenthesized disaccharide unit proximal to the dehydromannose or dehydromannitol residue.

In that each "R" can be H or a cation, the fifty-seven possible structures represent a significantly larger number of compounds, i.e., the acid and salt forms.

The basic structural formulae of the fifty-seven configurations are not put forth herein. However, these formulae can be deduced from formula III and Figures 6A, 6B and 6C by referring to the formulae II(1) - II(11) above.

Preferred compounds of the invention are the hexasaccharides. However, the preferred octasaccharides include octasaccharides having antiproliferative activity with smooth muscle cells which have the formula



wherein at least two of the six sugars in the middle -GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA- group include a sulfate; or a physiologically acceptable salt thereof.

Especially preferred among these are octasaccharides wherein at least two IdoA-GlcNY units are

$$\text{IdoA-GlcNS-IdoA-GlcNS}$$


Thus, preferred octasaccharides of the invention include any of the following octasaccharides,

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their pharmaceutically acceptable salts and mixtures of two or more of such octasaccharides and their salts

- IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5);
 2S 6S 2S 6S 2S 6S 2S 6S
 5 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5);
 2S 6S 2S 6S 2S 6S 6S
 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5);
 2S 6S 2S 6S 2S 2S 6S
 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5);
 10 2S 6S 2S 6S 6S 2S 6S
 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5);
 2S 6S 2S 2S 6S 2S 6S
 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5); and
 2S 6S 6S 2S 6S 2S 6S
 15 IdoA-GlcNS-IdoA-GlcNS-IdoA-GlcNS-IdoA-Man(2,5).
 2S 2S 6S 2S 6S 2S 6S

Statement of Utility

- The oligosaccharide compositions of the invention
 20 are useful in therapeutic applications for treatment of
 conditions or diseases which are characterized by exces-
 sive and destructive smooth muscle cell proliferation.
 These conditions frequently occur where the subject has
 been exposed to trauma, such as in the case of surgical
 25 patients. The trauma caused by wounds or surgery results
 in vascular damage and secondary smooth muscle cell pro-
 liferation, which secondary proliferation results in
 vascular resenosis. This undesirable result can occur
 after vascular graft surgery, heart transplantation, bal-
 30 loon or laser angioplasty, arterial traumatic injury,
 postsurgical repair of muscular arteries, long-term
 in-dwelling of arterial catheters, invasive arterial
 diagnostic procedures, kidney, lung or liver transplants,
 coronary artery bypass surgery, carotid artery bypass
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surgery, femoral popliteal bypass surgery, and intracranial arterial bypass surgery.

In addition to secondary smooth muscle cell proliferation events occurring as a result of trauma, certain diseases are associated with unwanted vascular proliferation, although in these cases, too, it is assumed that some internal unknown injury has caused the secondary result. These disease states include Goodpasture syndrome, acute glomerulonephritis, neonatal pulmonary hypertension, asthma, congestive heart failure, adult pulmonary hypertension, and renal vascular hypertension.

For all these diseases and conditions, administration of suitable amounts of the compositions of the invention is useful in treatment. Administration is by typical routes appropriate for polysaccharide compositions, and generally includes systemic administration, such as by injection. Particularly preferred is intravenous injection, as continuous injection over long time periods can be easily continued. Typical dosage ranges are in the range of 0.1-10 mg/kg/hr on a constant basis over a period of 5-30, preferably 7-14, days. Particularly preferred dosage is about 0.3 mg/kg/hr, or, for a 70 kg adult, 21 mg/hr or 540 mg/day.

Other modes of administration are less preferred but may be more convenient. Injection subcutaneously at a lower dose or administered orally at a slightly higher dose than intravenous injection, or by transmembrane or transdermal or other topical administration for localized injury may also be effective. Localized administration through a continuous release device, such as a supporting matrix, perhaps included in a vascular graft material, is particularly useful where the location of the trauma is accessible.

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Formulations suitable for the foregoing modes of administration are known in the art, and a suitable compendium of formulations is found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, latest edition.

The compositions of the invention may also be labeled using typical methods such as radiolabeling, fluorescent labeling, chromophores or enzymes, and used in a competitive assay for the amount of antiproliferative component in a biological sample. Suitable protocols for competitive assays of analytes in biological samples are well known in the art, and generally involve treatment of the sample, in admixture with the labeled competitor, with a specific binding partner which is reactive with the analyte such as, typically, an immunoglobulin or fragment thereof. The antibodies prepared according to the invention are useful for this purpose. The binding of analyte and competitor to the antibody can be measured by removing the bound complex and assaying either the complex or the supernatant for the label. The separation can be made more facile by preliminary conjugation of the specific binding partner to a solid support. Such techniques are well known in the art, and the protocols available for such competitive assays are too numerous and too well known to be set forth in detail here.

The antibodies of the invention are useful in immunoassays, not only of the type described above involving competition between labeled composition and the analyte antiproliferation factor in the sample, but also for direct immunoassay for the factor. Alternate protocols involving direct assays are also of wide variety and well known. Typically, the analyte bound to antibody is detected by means of an additional reactive

partner which bears a label or other means of detection. Thus, in typical sandwich assays, for example, the binding of the antibodies of the invention to analyte can be detected by further reaction with a labeled
5 preparation of these same antibodies or by labeled antibody immunoreactive with this preparation by virtue of species differences.

The antibodies of the invention can also be formulated into pharmaceutical compositions and used to
10 stimulate the growth of smooth muscle cells in subjects for which this result is desirable.

EXAMPLES

The following examples are put forth so as to
15 provide those of ordinary skill in the art with a complete disclosure and description of how to make the compounds and compositions of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to insure
20 accuracy with respect to numbers used (e.g., amounts, temperature, etc.), but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, and pressure is at or near
25 atmospheric.

Example 1

Preparation of Hexa- and Octasaccharide

Heparin Fragments

30 To 20 g of heparin dissolved in 160 ml water was added 690 mg solid NaNO_2 to give a final HONO concentration of 50 mM in the deamination mixture. Approximately 70 ml 6 M HCl was added dropwise while the
35 mixture was stirred with a magnetic stirrer. The pH

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dropped slowly to 1.5 and was maintained at 1.5 by dropwise addition of either 6 M HCl or 2 M Na₂CO₃. Initially, the addition of acid caused the reaction mixture to turn yellow, but, as the reaction reached completion (about 6 min, when N₂ evolution ceased), the solution became clear. When the reaction was complete, 2 M Na₂CO₃ was added to bring the final pH up to 8.5. A fine white precipitate which sometimes appeared was removed by centrifugation and the supernatant was decanted, degassed under vacuum, and then loaded directly onto a BioGel P10 column.

For the BioGel chromatography, two columns were connected in tandem, each approximately 5 cm in diameter, 128 cm in length, were packed with a total of 5 l. of BioGel P10. The columns were prepared and run in 0.5 M NH₄HCO₃ at a flow rate of 0.7 ml per min. The deamination mixture was loaded onto the column in the smallest possible volume (less than 160 ml). Fractions of 18 ml were collected and analyzed by the carbazole procedure. Fractions in individual peaks were combined and dried by extensive lyophilization to remove the NH₄HCO₃. Peaks containing mixtures of di-, tetra-, hexa-, octa-, deca-, and higher oligosaccharides were obtained, with the higher oligosaccharides eluting early and the disaccharides eluting last.

Example 2

Effect on Smooth Muscle Proliferation

Solutions to be tested were made up in "complete medium", which is DMEM medium containing 10% fetal calf serum and penicillin/streptomycin.

Bovine smooth muscle cells (SMC) were isolated from bovine pulmonary artery by the method of Ross, R.J., Cell Biol (1971) 172-186. SMC from passage 3-10 were plated

at 350-700 cells per well in 96-well microtiter plates in the medium above and allowed to attach for 2-4 hr. The complete medium was replaced with DMEM supplemented with 0.1% fetal calf serum, and the cells were incubated for
5 an additional period of about 24 to 72 hr to arrest cell growth. The low-serum medium was then replaced with complete medium containing the test samples.

The cells were allowed to grow for up to 7 days with replicate plates sampled at regular intervals. Cell
10 number was determined by removing the medium and washing the cells with phosphate-buffered saline, adding 75-150 ul lysis buffer, and assaying for lactate dehydrogenase (LDH) activity, as described by Brandley, B., et al., J Biol Chem (1987) 262:6431. The activity of
15 LDH is proportional to cell number.

The results of one such assay on oligosaccharides ranging in size from tetra- to tetradecasaccharide fractions are shown in Figure 2. These results show that hexasaccharide and octasaccharide fractions are active in
20 the antiproliferative assay; the tetrasaccharide fraction appears substantially less active. While high molecular weight fragments are also active in this assay, fragments of hexasaccharide length and longer have comparable activity to heparin on a concentration weight/volume
25 basis and shorter oligosaccharides are more conveniently amenable to de novo synthesis. Hence, the minimal unit capable of antiproliferative activity is of interest.

As shown in Figure 2, 80% inhibition of proliferation is found at concentrations of the octasaccharide
30 fraction as low as 15 ug/ml. Comparable inhibition is shown by the hexasaccharide fraction at about 60 ug/ml.

Example 3
Anion Exchange Separation of
Hexasaccharides and Octasaccharides

The hexasaccharide and octasaccharide fractions
5 obtained according to Example 1 were subjected to anion
exchange chromatography on a 1 x 7 cm column of DEAE-
toyopearl packed in 0.1 M NH_4HCO_3 and developed with a
linear gradient from .01 M to 1.0 M NH_4HCO_3 (total volume
= 600 ml). Approximately 20 mg of each oligosaccharide
10 mixture was loaded onto the column.

The results are shown in Figure 3A and 3B for the
hexasaccharides and octasaccharides respectively. The
eluate was divided into six fractions of equivalent mass
as shown in Figures 3A and 3B and assayed according to
15 the method of Example 2 for ability to inhibit smooth
muscle proliferation. The results of this assay are
shown in Figures 4A and 4B.

As shown in Figure 4A, the ability to inhibit
smooth muscle proliferation appears correlated with
20 charge, as the highest charged fractions are considerably
more effective. Fractions eluting early in either column
do not appear to have substantial antiproliferative
activity. However, fractions with high affinity for the
anion exchanger are quite effective. For example, in the
25 case of the hexasaccharide mixture, concentrations of
roughly 75 ug/ml of any of the four highest charged
fractions gave inhibition of smooth muscle proliferation
in the assay equivalent to 60% of the maximum inhibition
obtainable with commercial heparin; the highest charged
30 four fractions of the octasaccharide anion exchange
separation were capable of approximately 60-80%
inhibition at 15-30 ug/ml.

The DEAE-toyopearl chromatography was run on a
larger scale using a 5 cm x 27 cm column packed in 0.3 M
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NH₄HCO₃. Up to 3 g of oligosaccharide mixture was loaded onto this column and the column was washed successively with 2 l volumes of 0.3 M, 0.5 M, .06 M, 0.9 M, and 1.2 M NH₄HCO₃. The fraction emerging in 0.9 M NH₄HCO₃,
5 equivalent to the most highly sulfated pool from the smaller column, is recovered in yields of approximately 150 mg/g of starting oligosaccharide mixture.

Example 4

10 Reverse Phase Ion Pairing HPLC

The unresolved hexasaccharides and the hexasaccharide fraction which is of the highest charged character obtained from the DEAE-Toyopearl column in Example 3 were subjected to reversed phase ion paring
15 HPLC as described by Guo, Y., et al., Anal Biochem (1988) 168:54-62. The elution patterns for these procedures are shown in Figures 5A and 5B.

Figure 5A shows the elution profile from the highest charged fragment of DEAE-Toyopearl; Figure 5B
20 shows the results using the total hexasaccharide fraction. As a comparison of these profiles will indicate, the charge separated fraction is a greatly simplified mixture. The individual components of this simplified mixture are expected to have antiproliferative
25 activity.

The more highly charged fragments generally show (as compared with less highly charged fragments and/or commercial heparin) (1) a greater ability to inhibit the proliferation of smooth muscle cells and (2) a lesser
30 ability to act as an anticoagulant. Further fractionation/separation processing can be carried out which improve factors (1) and (2) and also simultaneously aid in eliminating fragments which include
35 oligosaccharides sulfated at the 3-position. It is

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pointed out that in order for a glucosamine to have anticoagulant activity it must be sulfated at the 3-position. Preferred oligosaccharide fragments of the invention possess characteristic (1) and (2) and (3) are highly charged and (4) include a very low (or no) amounts of saccharides sulfated at the 3-position as compared with fragments of commercial heparin. In order to obtain such preferred oligosaccharides, it is preferable to produce them synthetically rather than obtain them from digestion of heparin.

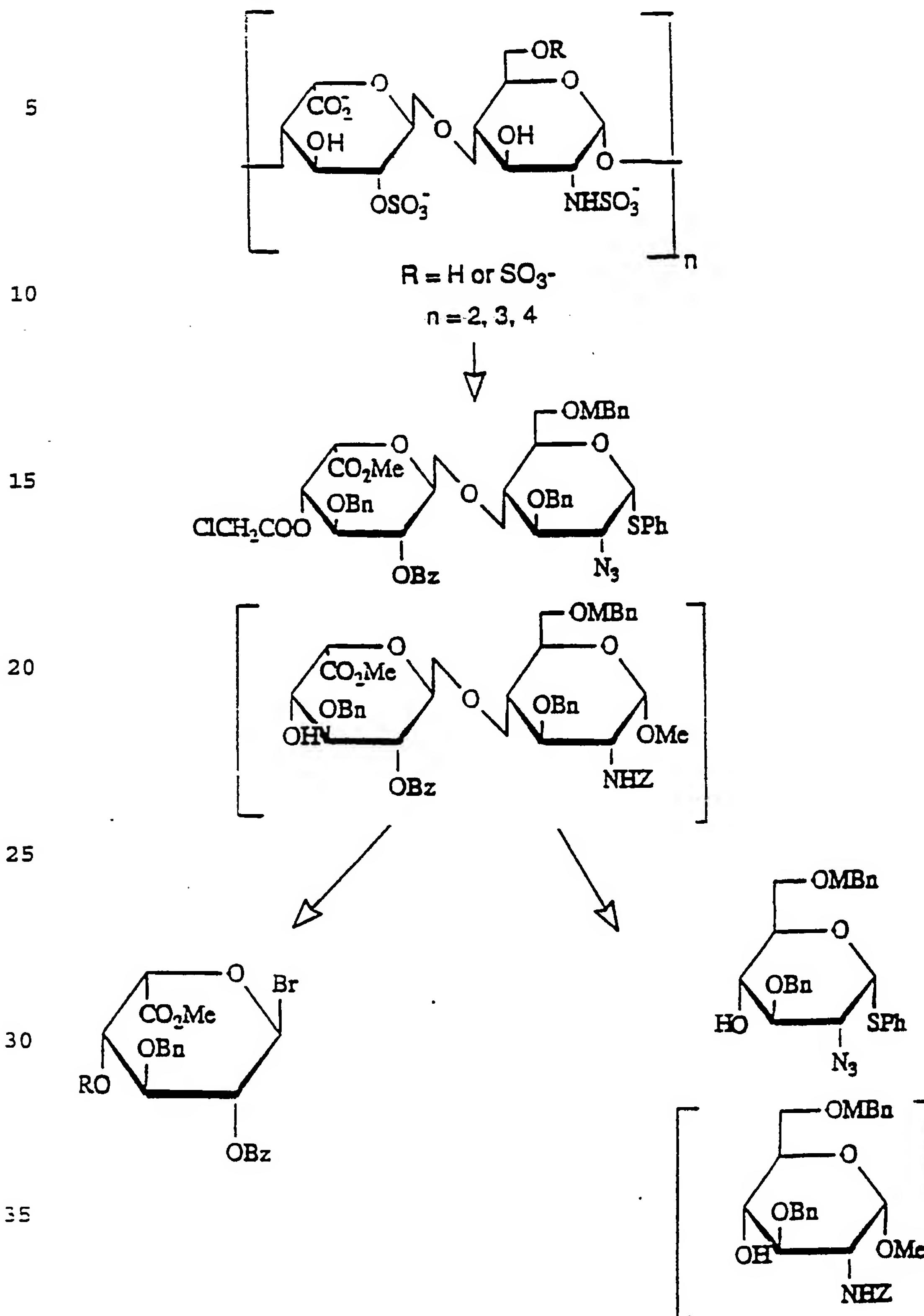
Example 5

Synthesis of Preferred Oligosaccharides

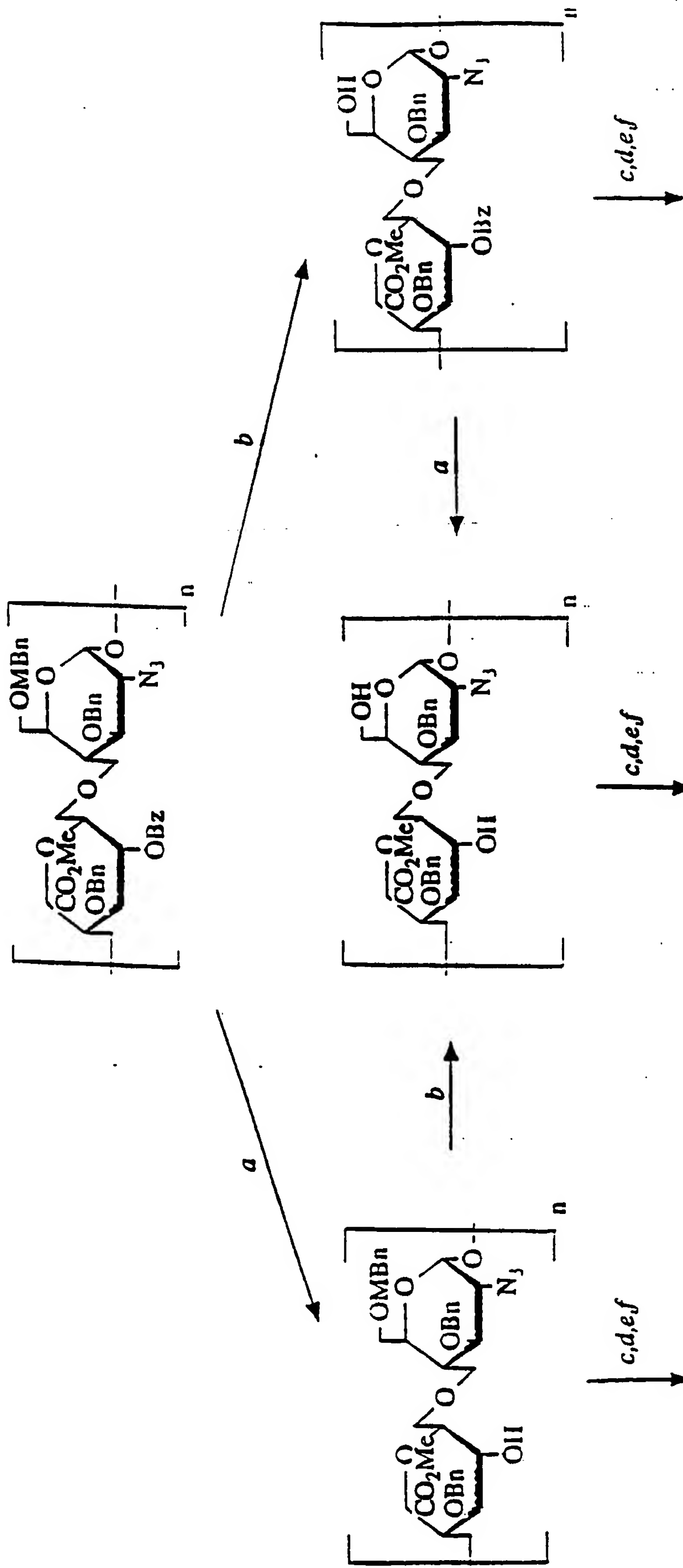
As indicated above, the oligosaccharides of the invention which are particularly preferred have a number of distinct characteristics such as greater ability to inhibit proliferation of smooth muscle cells, lesser ability to act as an anticoagulant, high degrees of sulfation, and lack of sulfation at the 3-position. Although it is possible to obtain such particularly preferred oligosaccharides by the digestion of heparin and thereafter separation of the fragments obtained in accordance with methods described above, it is preferable to obtain such particularly preferred fragments using chemical synthesis methodologies. Chemical synthesis methodologies makes it possible to obtain highly pure reaction products all of which have the same structure and therefore characteristics. The following is a flow diagram which shows the synthesis of particularly preferred oligosaccharides. At the end of the structural synthesis schemes put forth below, a written description is provided which describes methods of carrying out such synthesis.

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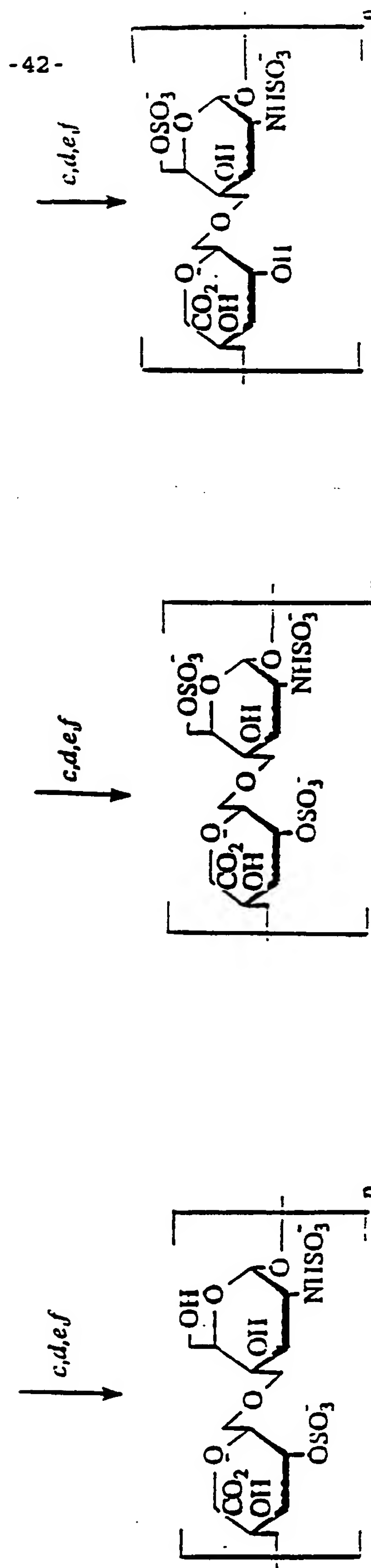
Schematic Structural Retrosynthesis for Making
Particularly Preferred Oligosaccharides



Deprotection and Sulfation of Particularly Preferred oligosaccharides



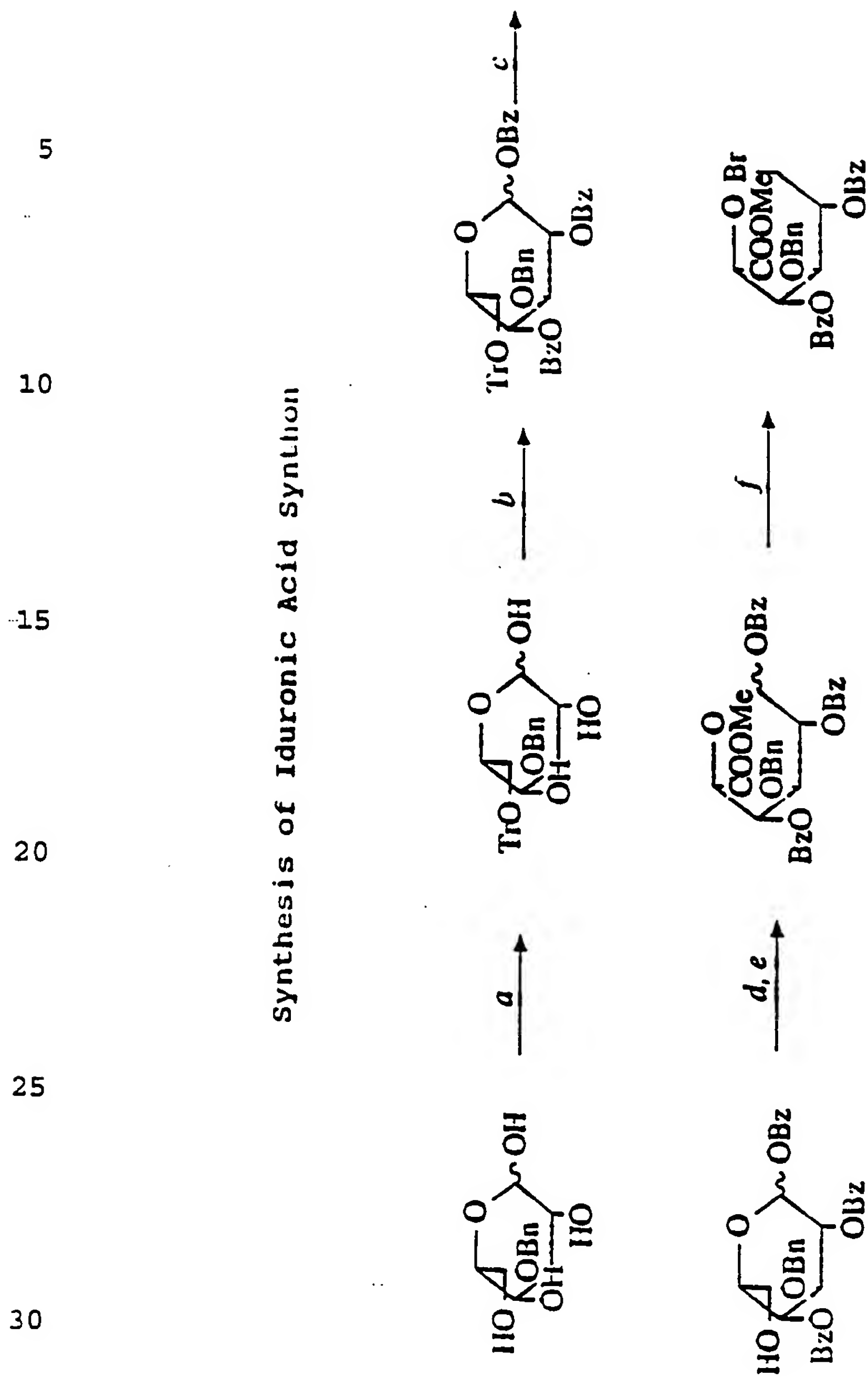
Deprotection and Sulfation of Particularly Preferred Oligosaccharides (cont'd)



a) debenzoylation; b) de-methoxybenzoylation; c) O-sulfation
d) catalytic hydrogenation; e) deesterification; f) N-sulfation

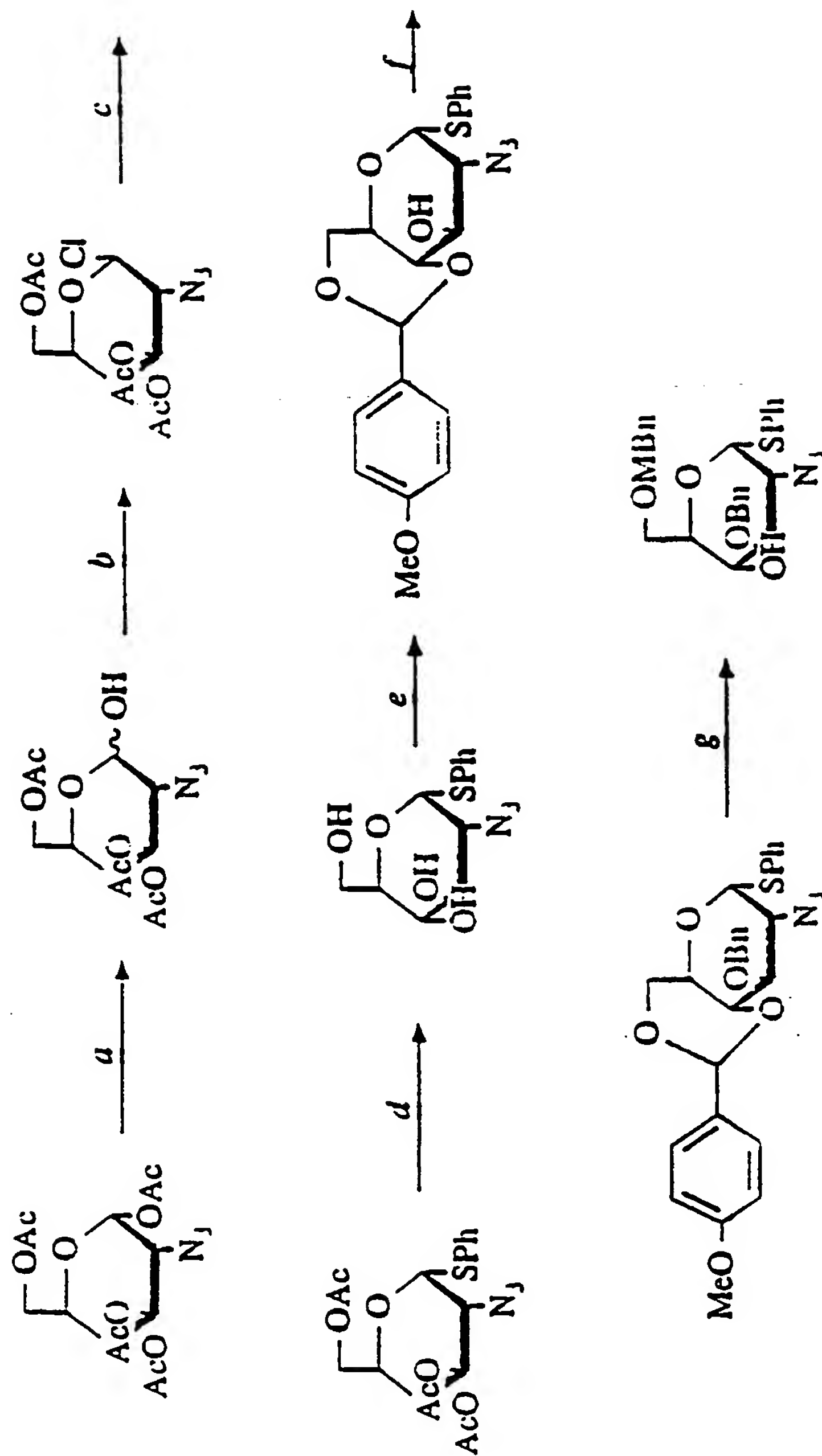
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Synthesis of Iduronic Acid Synthron



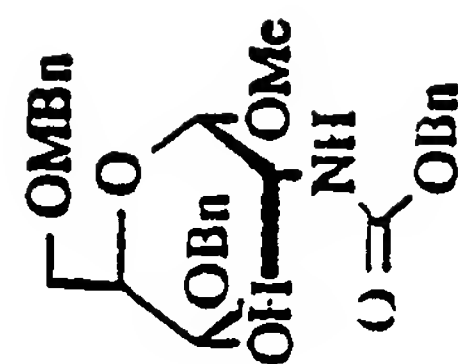
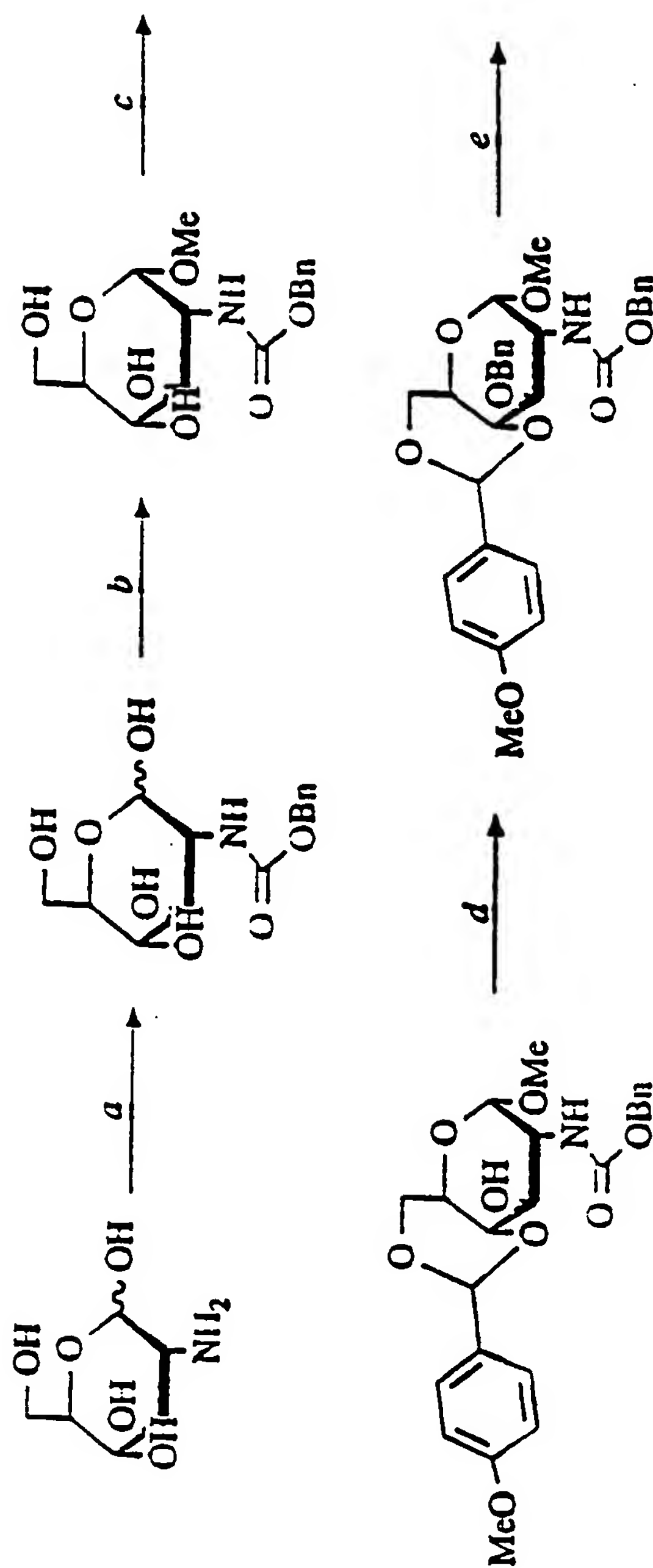
a) TrCl , Pyr; b) BzCl , Pyr; c) 80% AcOH ; d) CrO_3 , acetone; e) CH_2N_2 , CH_2Cl_2 ; f) TiBr_4

Synthesis of Internal Glucosamine Synthons



a) hydrazine acetate, DMF; b) $SOCl_2$, DMF, CH_2Cl_2 ; c) $NaSPh$, DMF; d) $NaOMe$, $MeOH$; e) *p*-methoxybenzaldehyde dimethylacetal, CSA, $MeCN$; f) $BnBr$, NaH , DMF; g) $NaCNBH_3$, TFA, DMF

Synthesis of Reducing Terminal Glucosamine Synthons



a) ClCOOBn, NaHCO₃, water; b) MeOH, HCl; c) p-methoxybenzaldehyde dimethylacetal, CSA, MeCN; d) BnBr, Ba(OH)₂, DMF; e) NaCNBH₃, TFA, DMF

Synthesis of Disaccharide Synthons

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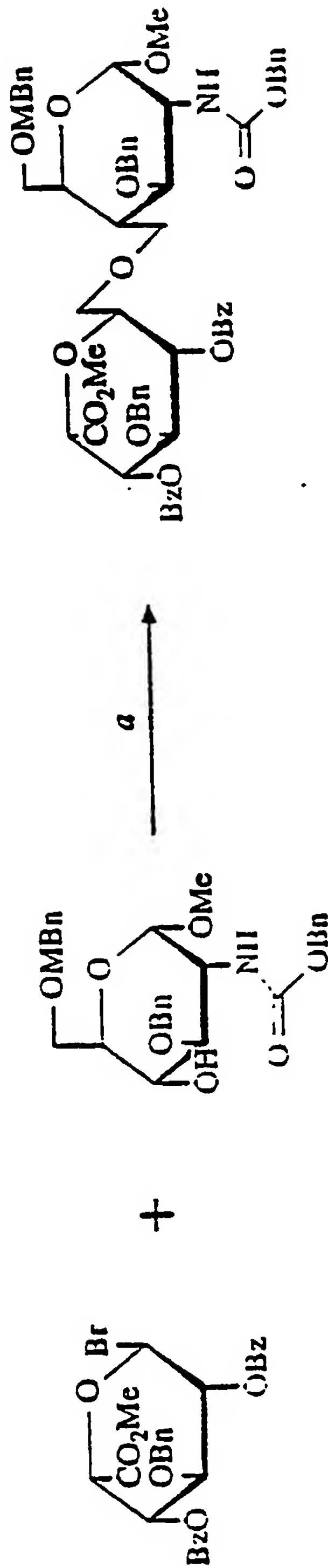
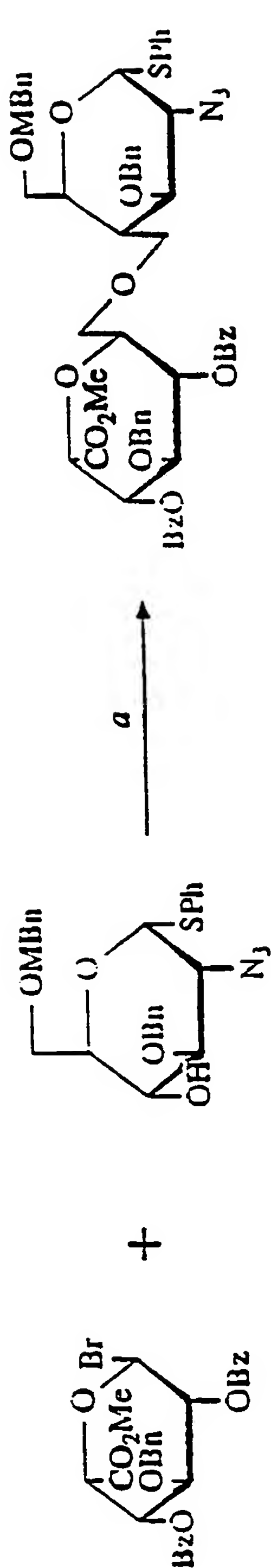
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a) AgOTf, collidine, CH_2Cl_2

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Functionalization of the Reducing Terminal Disaccharide Synthron

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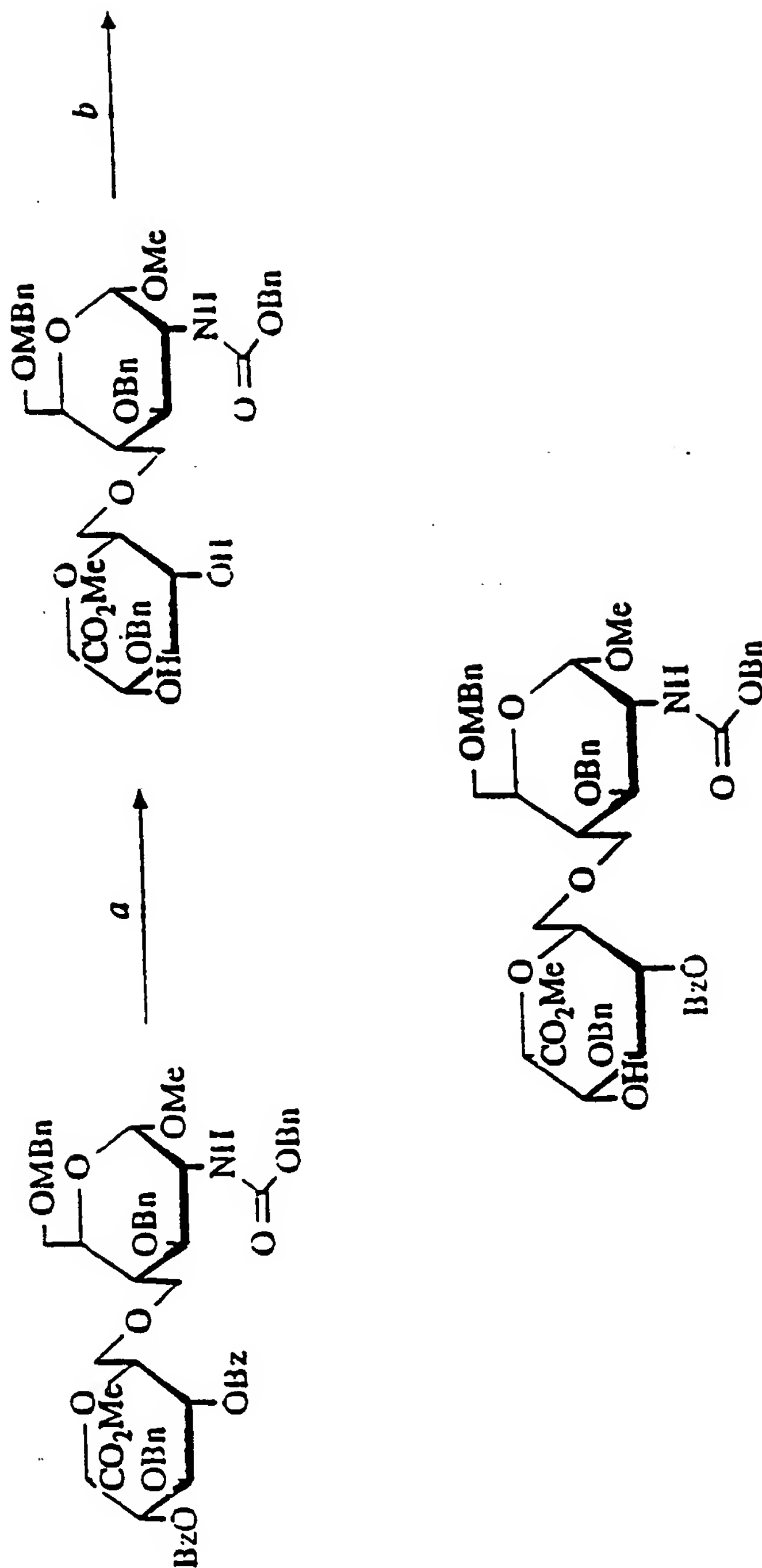
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*a) NaOMe, MeOH; b) BzCl, Pyr*

Functionalization of the Internal Disaccharide Synthons

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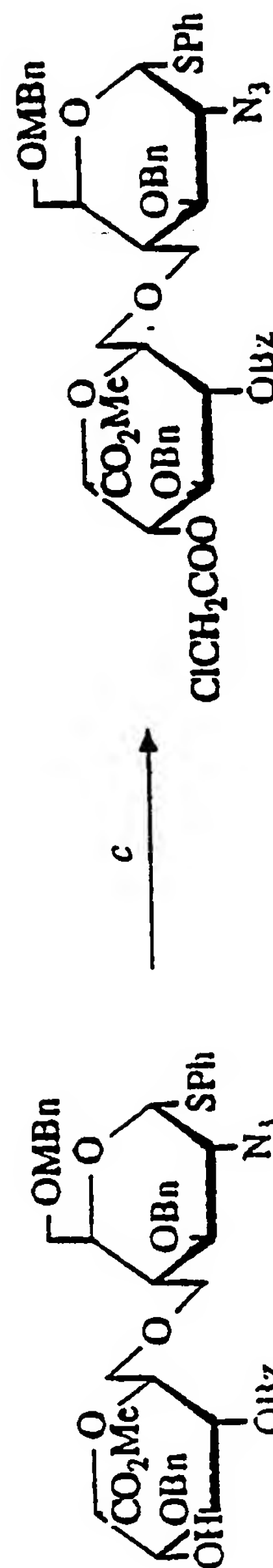
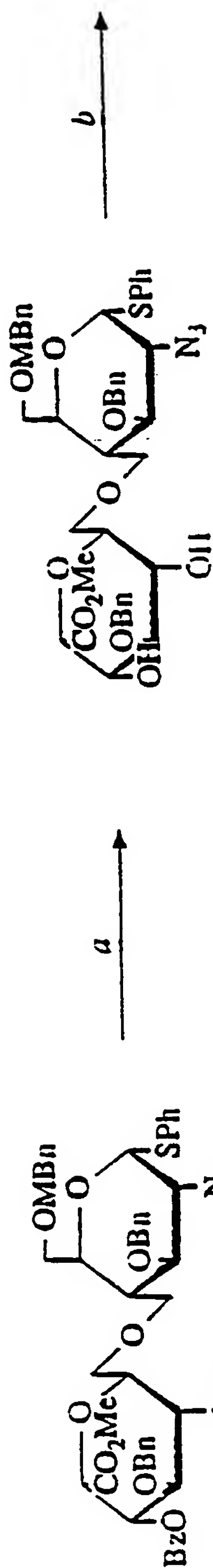
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a) NaOMe, MeOH; *b*) BzCl, Pyr; *c*) chloroacetyl chloride, Pyr, CH₂Cl₂

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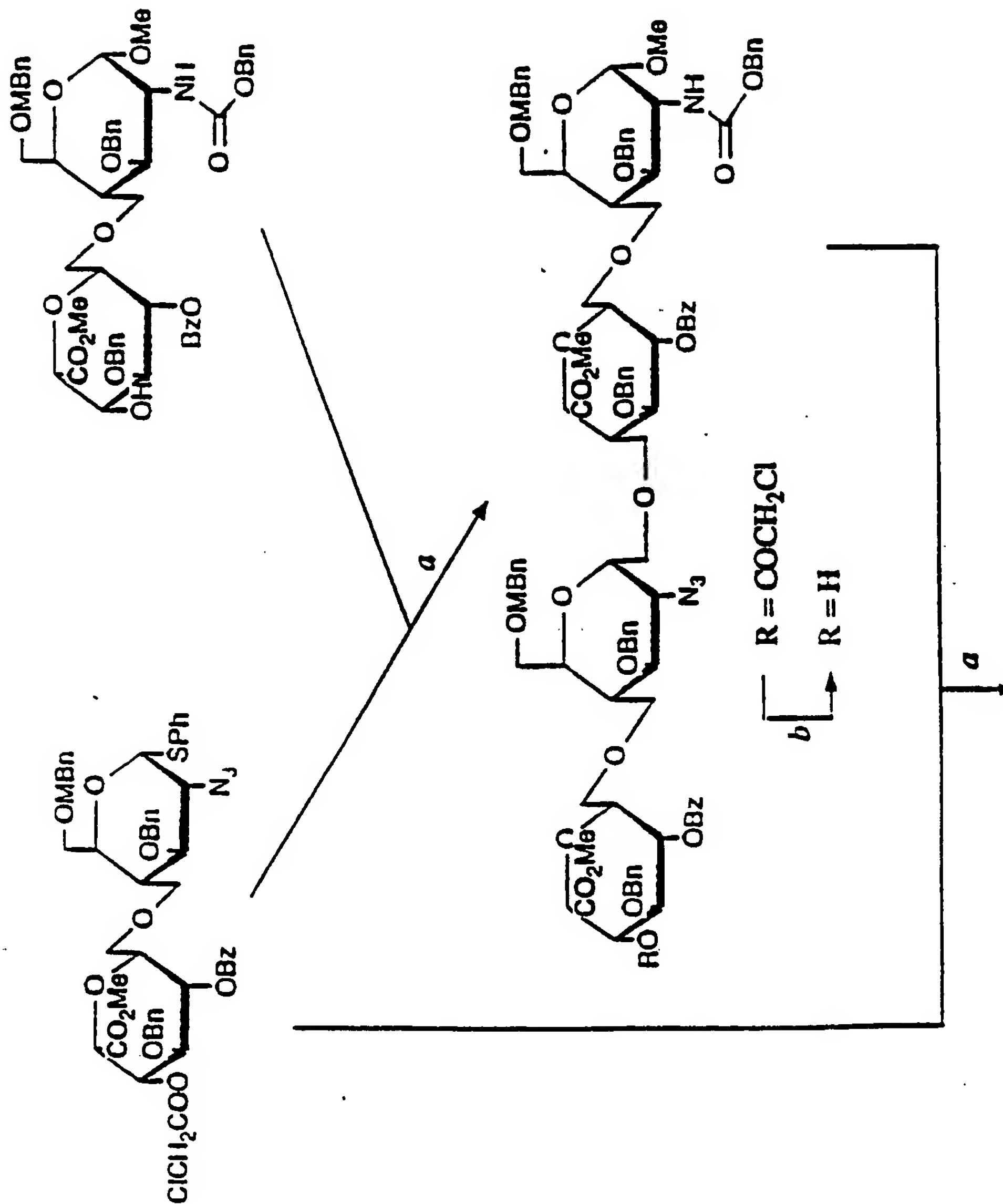
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Synthesis of Protected Hexasaccharide



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Synthesis of Protected Hexasaccharide (cont'd)

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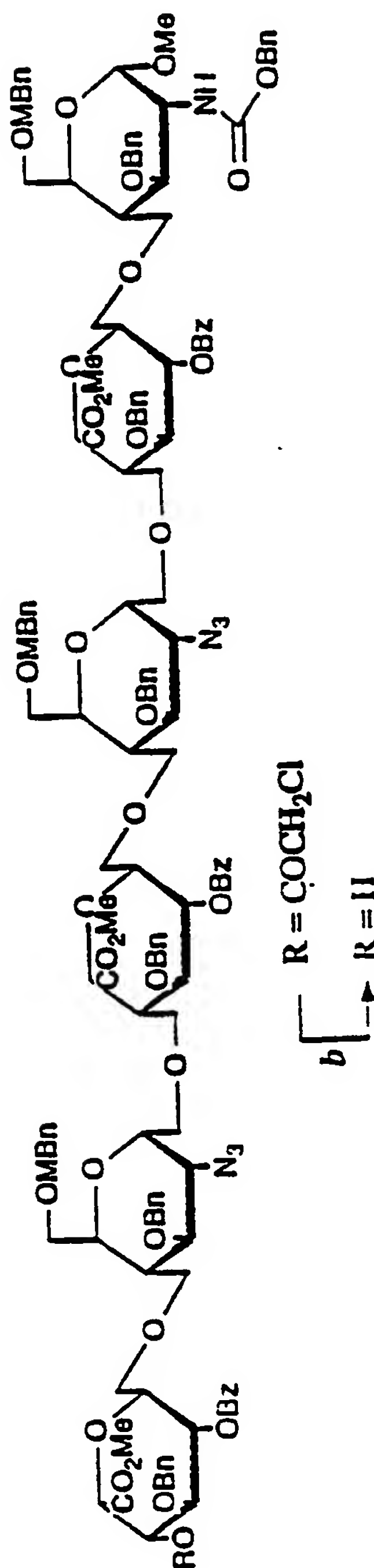
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a) DMTST, ether- CH_2Cl_2 ; b) thiourea, EtOAc-MeOH

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Retrosynthesis of Particularly Preferred Oligosaccharides

Oligosaccharides, regardless of their size or whether their glucosamine unit is sulfated at 0-6, can be retrosynthesized to a common protected disaccharide unit.

5 This disaccharide unit is open for chain extension in both directions; its thioglycoside function permits chain-extension towards the reducing end, whereas protection of its 0-4' position by the temporary chloracetyl group allows further chain-extensions towards
10 the non-reducing end. The amino groups of the target compounds are masked as azido groups, which assure advantageous stereocontrol in glycosylation reactions. Benzyl (Bn) groups are used as permanent blocking groups for the hydroxyls which are nonsulfated in the target
15 compounds, and the semi-permanent benzoyl (Bz) group is used for the OH's to be sulfated. The p-methoxybenzyl (MBn) group stands for hydroxyl groups the sulfation of which is optional in the target compounds.

The disaccharide synthon should be available by
20 glycosylating the protected 2-azido-2-deoxy-glucopyranosyl derivative with the iduronosyl bromide.

Though all the target oligosaccharides can be synthesized from this single disaccharide synthon, for the particular case of the reducing end this synthon can
25 be substituted by another disaccharide unit (shown in brackets), which bears a methyl glycoside at the reducing end, and an amino group which is protected by a benzyloxycarbonyl (Z) group. This disaccharide can be synthesized by coupling the same iduronic acid donor with
30 a glucosamine derivative shown in brackets.

This retrosynthesis involves a novel and particularly advantageous blocking group strategy, which, in contrast to previous syntheses of sulfated oligosaccharides, permits the preparation of oligosaccharides
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-52-

with different sulfation patterns from the same protected derivative.

The combination of an acyl-type (benzoyl) protecting group with the p-methoxybenzyl group allows specific deprotection and subsequent sulfation in any order, leading to structures which have sulfate groups in positions: a) masked by benzoyl groups in the protected derivative; b) masked by p-methoxybenzyl groups in the protected derivative; and c) masked by both benzoyl and p-methoxybenzyl groups in the protected derivative.

A further advantage of the use of the p-methoxybenzyl group is that if selective removal of this group is not required it can be removed by catalytic hydrogenation in the same step as the permanent benzyl groups, thereby reducing the number of required synthetic steps.

For the synthesis of the iduronic acid donor 3-O-benzyl-L-idose (van Boeckel, C.A.A. et al., Carbohydr Chem (1985) 4:293, incorporated herein by reference) was tritylated with trityl chloride in pyridine and the product, without isolation, was directly benzoylated by the addition of benzoyl chloride to the reaction mixture. The trityl group was removed by acid hydrolysis, and the primary hydroxyl group was oxidized by chromic acid, followed by esterification of the resulting carboxyl group with diazomethane. Conversion to the glycosyl bromide was achieved with titanium(IV)bromide.

For the synthesis of the glucosamine synthon 2-azido-2-deoxy-D-glucose peracetate (Paulsen, H. et al., Chem Ber (1978) 111:2334, incorporated herein by reference) was converted into the thioglycoside in three steps by selective deacetylation with hydrazine acetate (Excoffier, G. et al., Carbohydr Res (1978) 39:368, incorporated herein by reference), followed by conversion

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of the resulting hemiacetal into the chloride, and subsequent thioglycosidation. The acetyl groups were removed by Zemplen-deacetylation, and the 4,6-O-p-methoxybenzylidene acetal was prepared by an acetal-exchange reaction. The 3-OH group was benzylated, and the 4,6-O-acetal ring was reductively opened with NaCNBH₃-trifluoroacetic acid (Johansson, R. et al., J Chem Soc (1984) 1:2371, incorporated herein by reference).

The glucosamine synthon for the reducing end was synthesized by an analogous sequence from methyl 2-benzyloxycarbonylamino-2-deoxy- α -D-glucopyranoside (Heyns, K. et al., Chem Ber (1955) 88:188, incorporated herein by reference) by p-methoxybenzylidenation, benzylation, and reductive ring opening.

Coupling of the 2-azido- and 2-benzyloxycarbonylamino-2-deoxy-D-glucose derivatives with the same iduronosyl bromide was performed using silver triflate, in combination with collidine as a buffer of the reaction medium, and resulted in the disaccharides.

Both disaccharides were further functionalized to the disaccharide synthons. The compounds were debenzoylated and a single benzoyl group was introduced at the 0-2' position. In the case of the disaccharide thioglycoside derivative, the benzoylation was followed by chloroacetylation of the 0-4' position.

The two disaccharide synthons were coupled by using dimethyl(methylthio)sulfonium triflate (Fügedi, P. et al., Carbohydr Res (1986) 149:C9, incorporated herein by reference) (DMTST) to give the tetrasaccharide with the required α -interglycosidic linkage.

Selective removal of the chloroacetyl group followed by glycosylation with the same disaccharide donor would give the protected hexasaccharide, which can

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be sulfated and deprotected in different ways as discussed previously.

Additional methods of synthesis may become apparent to those skilled in the art upon reviewing the above disclosure by itself and/or in combination with U.S. Patent 4,943,630 issued July 24, 1990, which patent is incorporated herein by reference to disclose methods of synthesizing oligosaccharides.

While the present invention has been described with reference to specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalence may be submitted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto.

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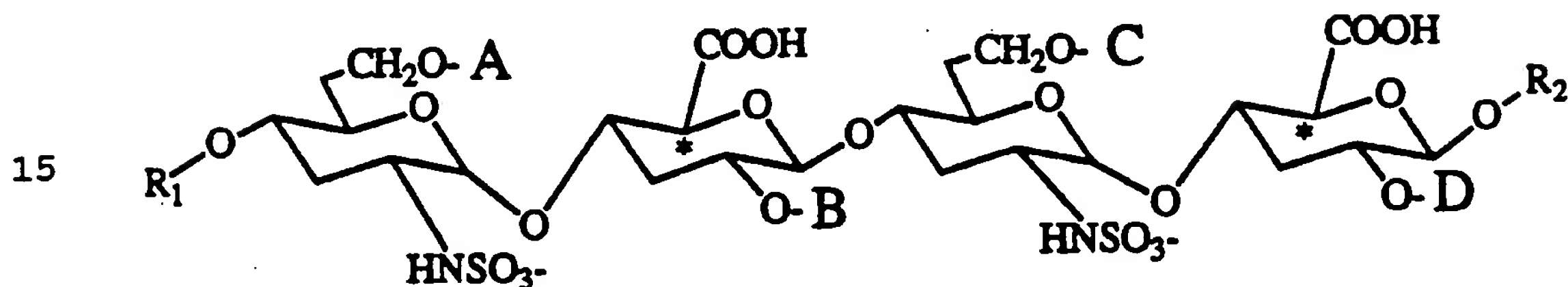
CLAIMS

What is claimed is:

- 5 1. A compound capable of inhibiting the proliferation of smooth muscle cells, having the following structural formula:

Formula I

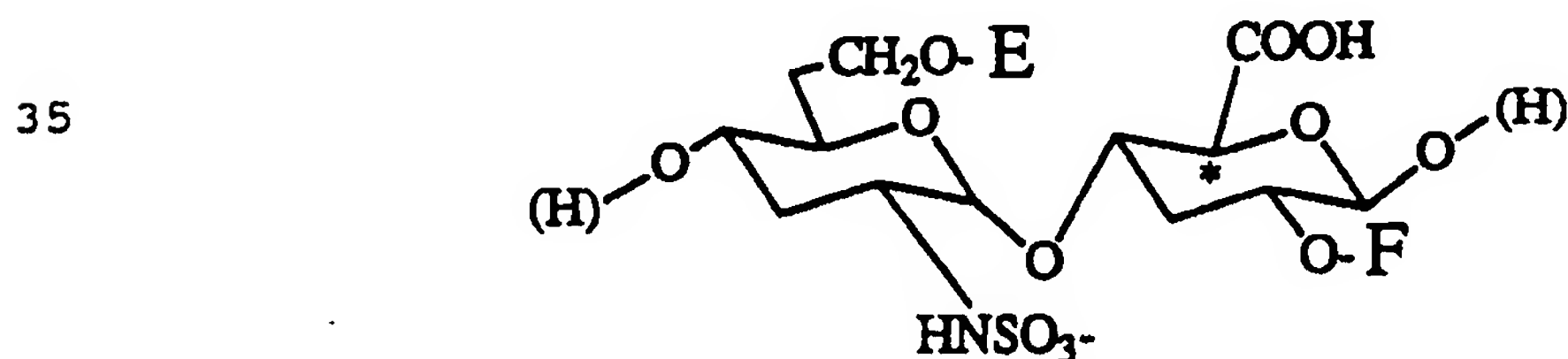
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- 20 wherein each of A, B, C, and D is independently H or SO_3R and each R is independently H or a cation, with the proviso that at least two of A, B, C or D is $-\text{SO}_3\text{R}$; noting that hydroxyl groups on the sugars have been omitted in Formulas I and I(a) for greater clarity and the * adjacent the carbon substituted with COOH indicates
- 25 any possible stereochemistry in either I or I(a); R_1 and R_2 are each independently hydrogen or one or more repeating units having the following structure:

Formula I(a)

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wherein when a unit of Formula I(a) is connected at one end, the hydrogen at that end is not present; and each of E and F is independently hydrogen or SO_3R and noting that the hydroxyl group on the 3-position on each sugar is present but has been omitted for greater clarity.

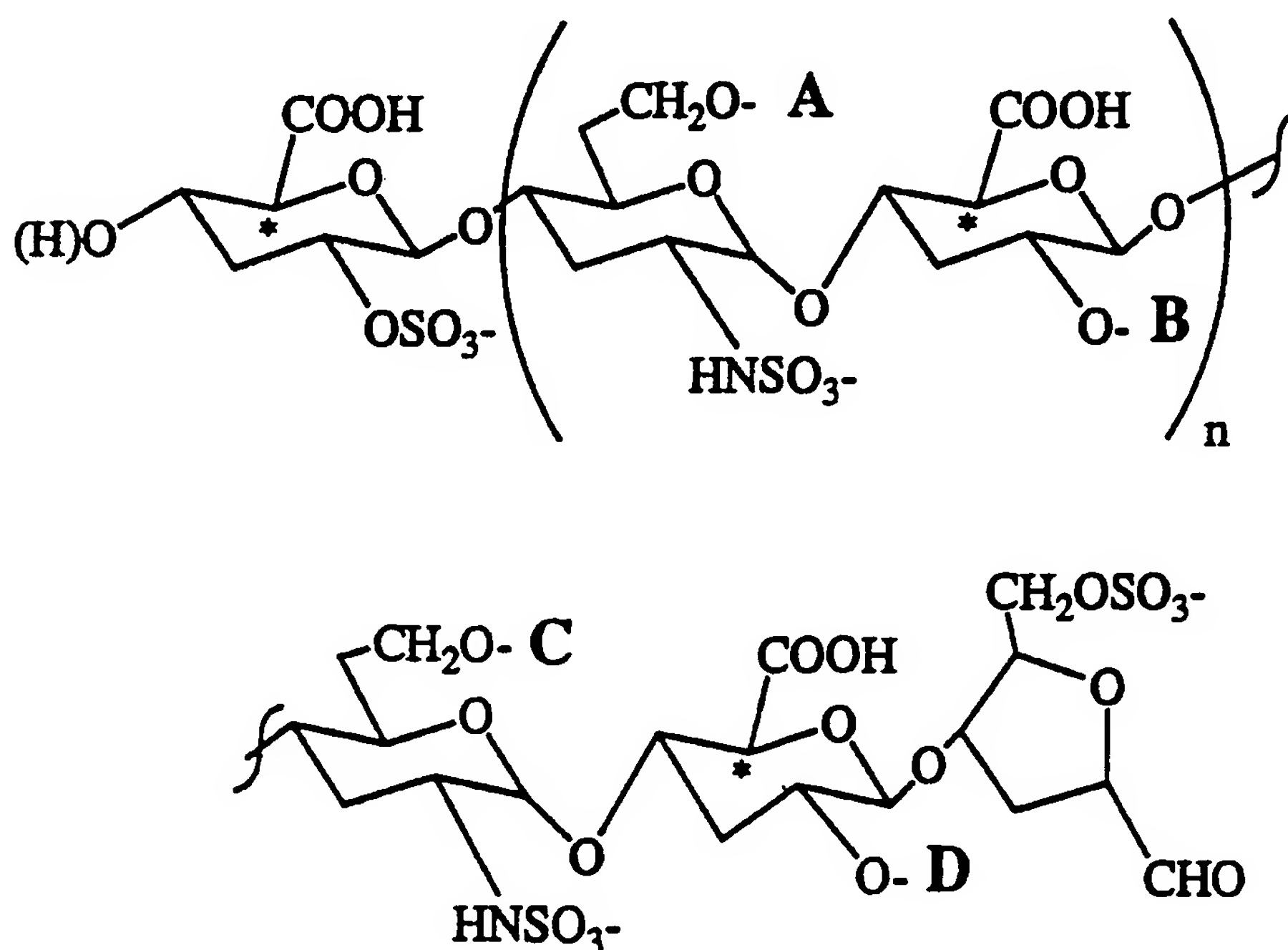
2. The compound of claim 1, wherein at least three of A, B, C or D is $-\text{SO}_3\text{R}$.

3. The compound of claim 2, wherein all of A, B, C and D are $-\text{SO}_3\text{R}$.

4. The compound of claim 1, wherein R_1 and R_2 are H, and each R is H, Na, K, Ca or NH_4 .

5. A mixture of hexasaccharide and octasaccharide compounds capable of inhibiting the proliferation of smooth muscle cells, the compound in the mixture having the following structural formula:

Formula III



wherein n is 1 or 2 and each of A, B, C and D is independently H or SO_3R , wherein each R is independently H or a cation, with the proviso that at least two of said A, B, C and D are SO_3R ; and wherein a hydroxyl group present on the 3 position of each sugar is present but has been omitted from the depiction; and wherein the asterisk on the carbons substituted with carboxyl indicates that the stereochemistry is not determined.

6. The mixture of claim 5, wherein at least three of A, B, C or D is $-\text{SO}_3\text{R}$.

7. The mixture of claim 5, wherein at least four of A, B, C or D is $-\text{SO}_3\text{R}$.

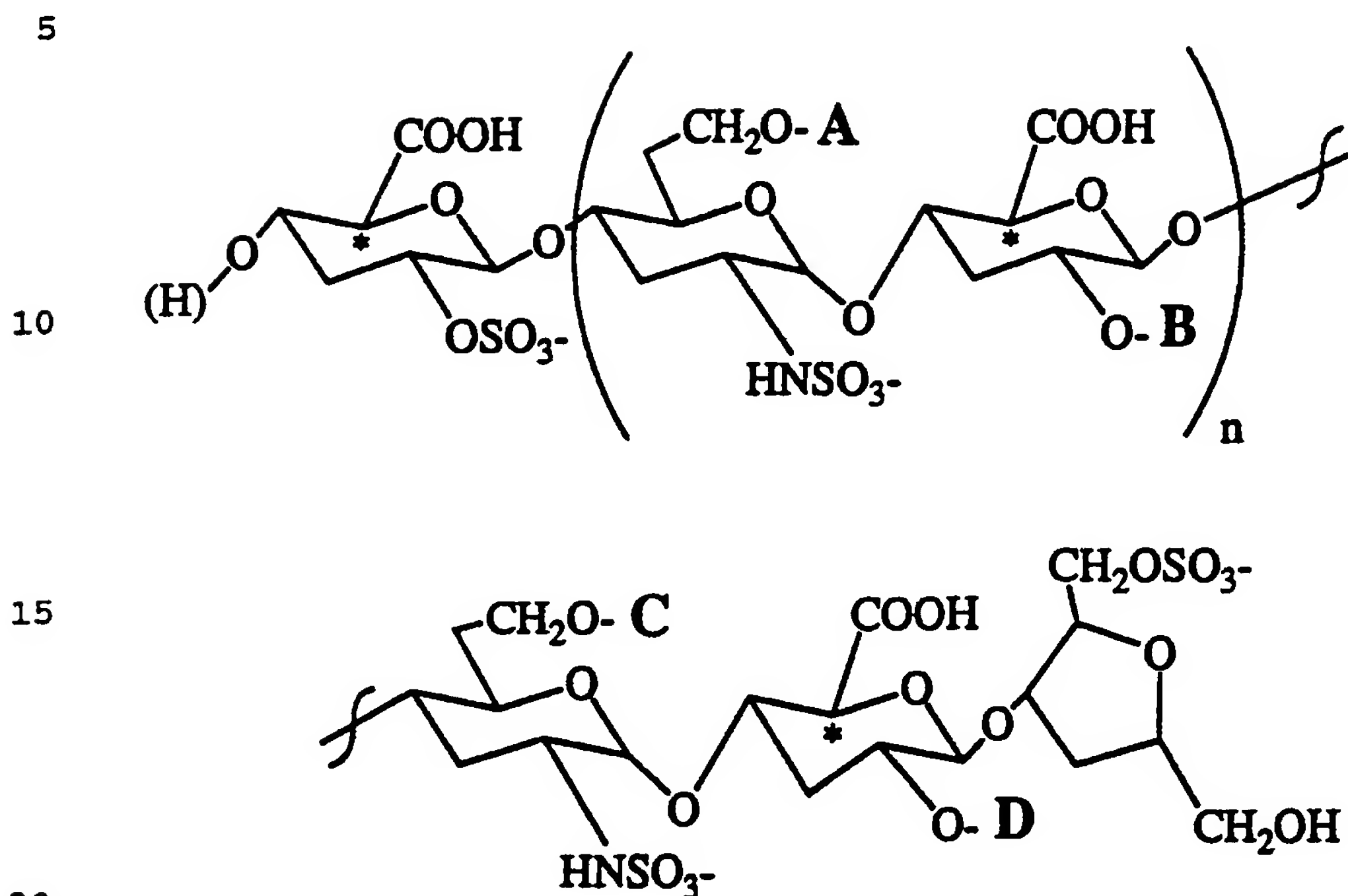
8. The mixture of claim 5, wherein at least five of A, B, C or D is $-\text{SO}_3\text{R}$.

9. The mixture of claim 5, wherein all of A, B, C and D are $-\text{SO}_3\text{R}$.

10. The mixture of claim 5, wherein each R is H, Na, K, Ca or NH_4 .

11. A mixture of hexasaccharide and octasaccharide compounds capable of inhibiting the proliferation of smooth muscle cells, the compound in the mixture having the following structural formula:

Formula III(a)



wherein n is 1 or 2 and each of A, B, C and D is independently H or SO_3R , wherein each R is independently H or a cation, with the proviso that at least two of said A, B, C and D are SO_3R ; and wherein a hydroxyl group present on the 3 position of each sugar is present but has been omitted from the depiction; and wherein the asterisk on the carbons substituted with carboxyl indicates that the stereochemistry is not determined.

12. The mixture of claim 11, wherein at least three of A, B, C or D is $-\text{SO}_3\text{R}$.

13. The mixture of claim 11, wherein at least four of A, B, C or D is $-\text{SO}_3\text{R}$.

-59-

14. The mixture of claim 11, wherein at least five of A, B, C or D is $-\text{SO}_3\text{R}$.

15. The mixture of claim 11, wherein all of A, B, C and D are $-\text{SO}_3\text{R}$.

16. The mixture of claim 11, wherein each R is H, Na, K, Ca or NH_4 .

17. A pharmaceutical composition useful in the treatment of conditions characterized by unwanted smooth muscle cell proliferation, comprising:

a pharmaceutically effective amount of a heparin digest derivative in the form of oligosaccharide fragments selected from the group consisting of hexasaccharide and octasaccharide or pharmaceutically acceptable salt thereof which fragments have an increased ability to inhibit smooth muscle cell proliferation and a decreased ability to act as an anticoagulant as compared with commercial heparin; and
a pharmaceutically acceptable carrier.

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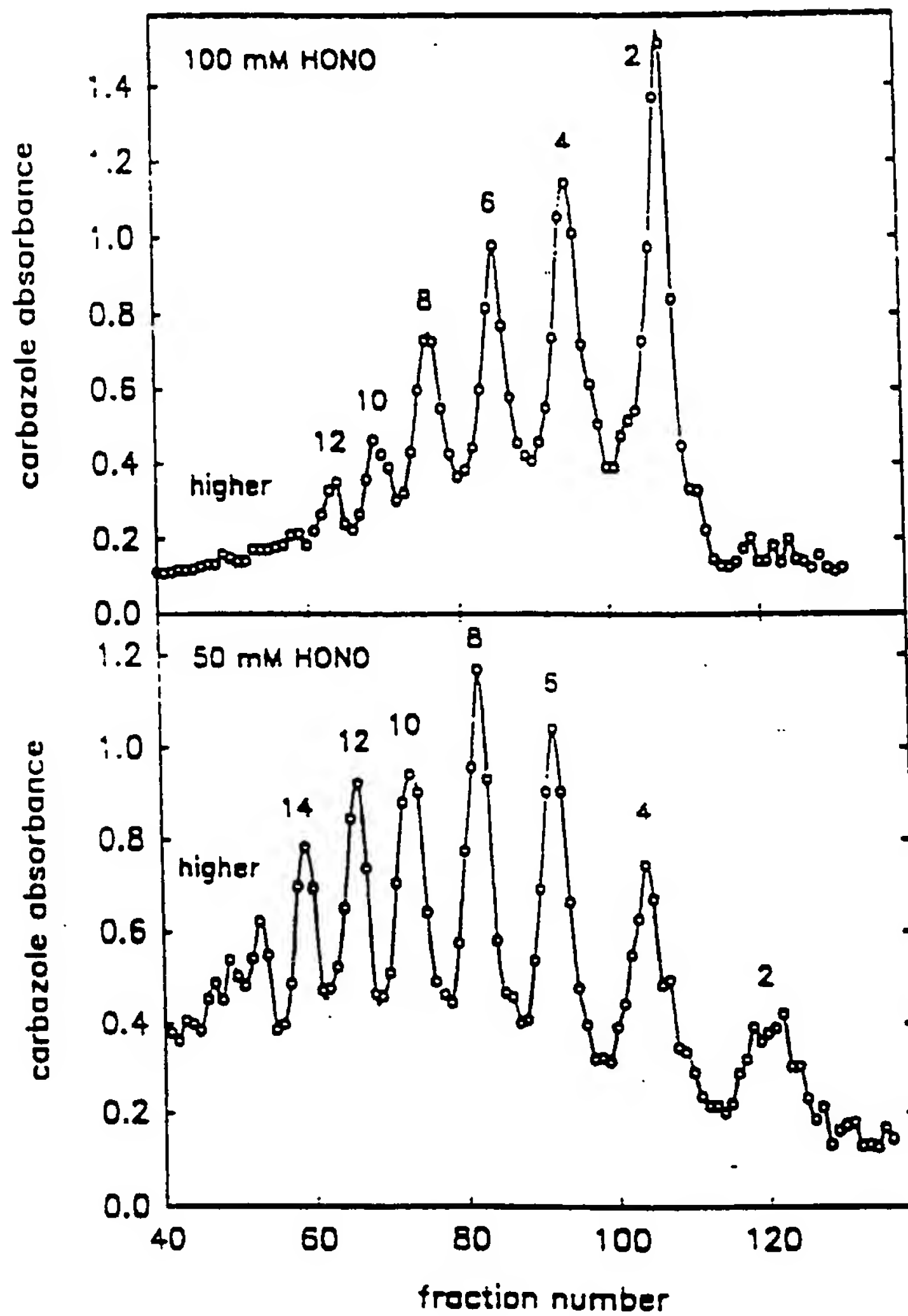
SEPARATION OF HEPARIN OLIGOSACCHARIDES
BY GEL FILTRATION CHROMATOGRAPHY

Fig. 1A

Fig. 1B

INHIBITION OF SMC GROWTH BY HEPARIN FRAGMENTS

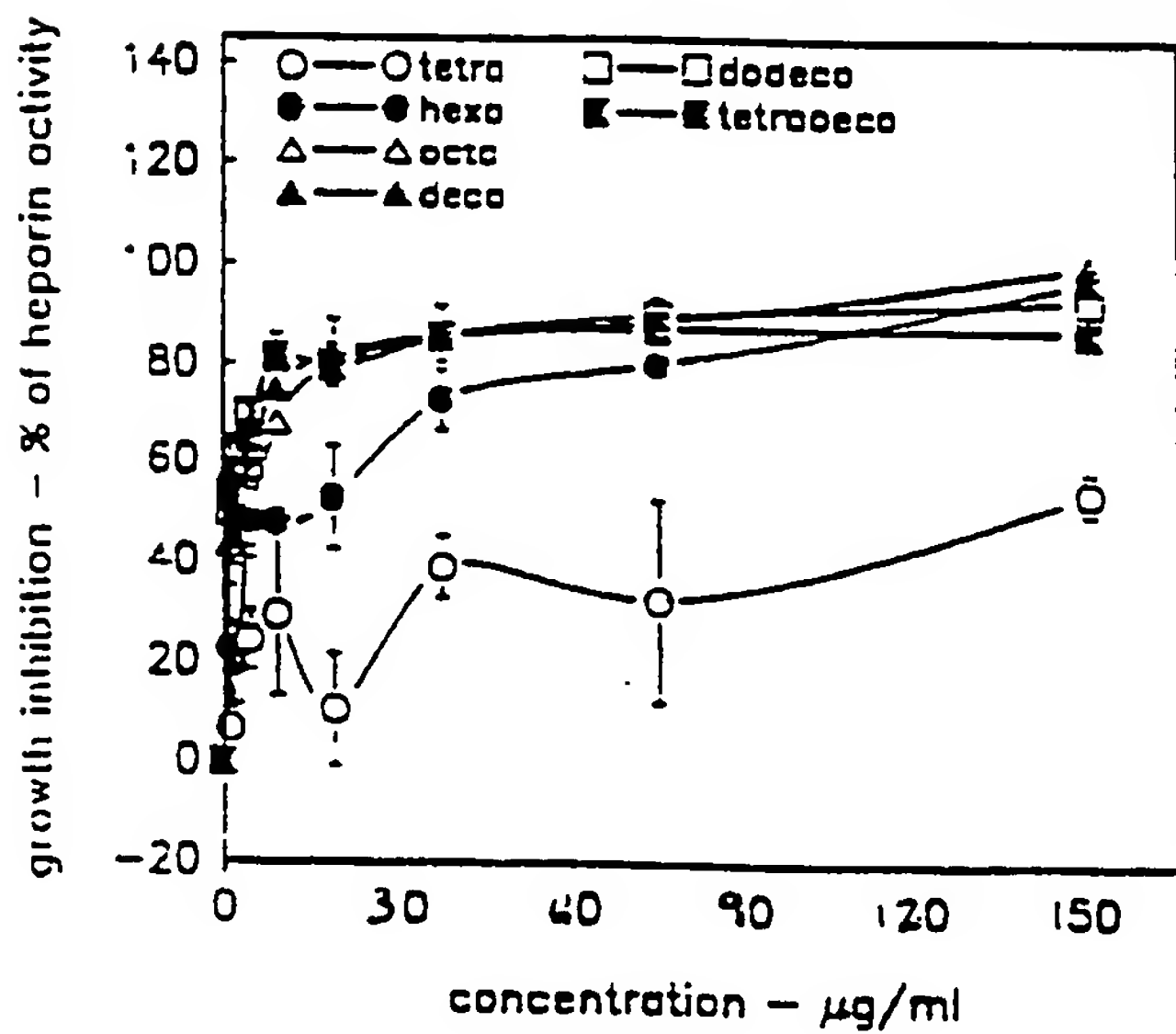


Fig. 2

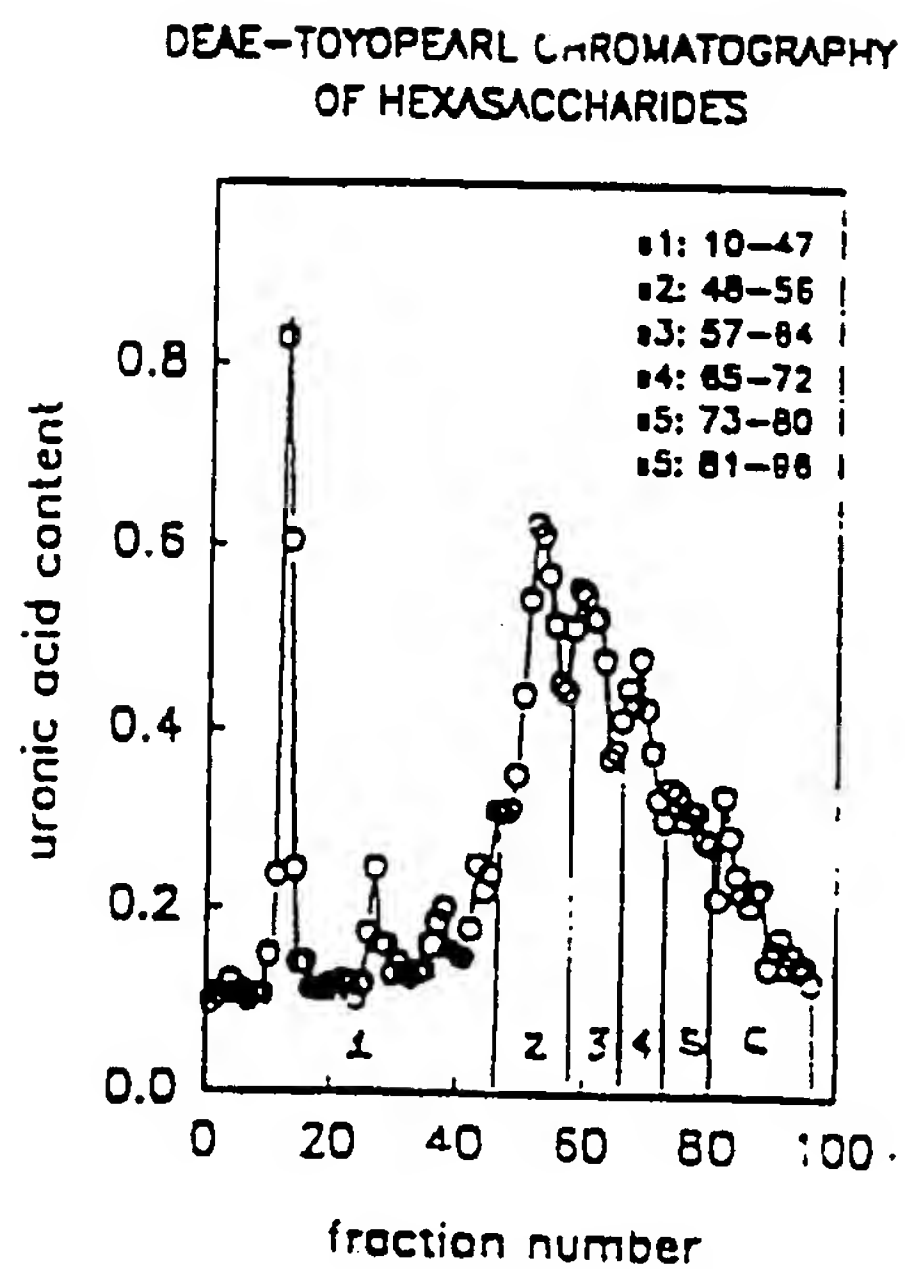


Fig. 3A

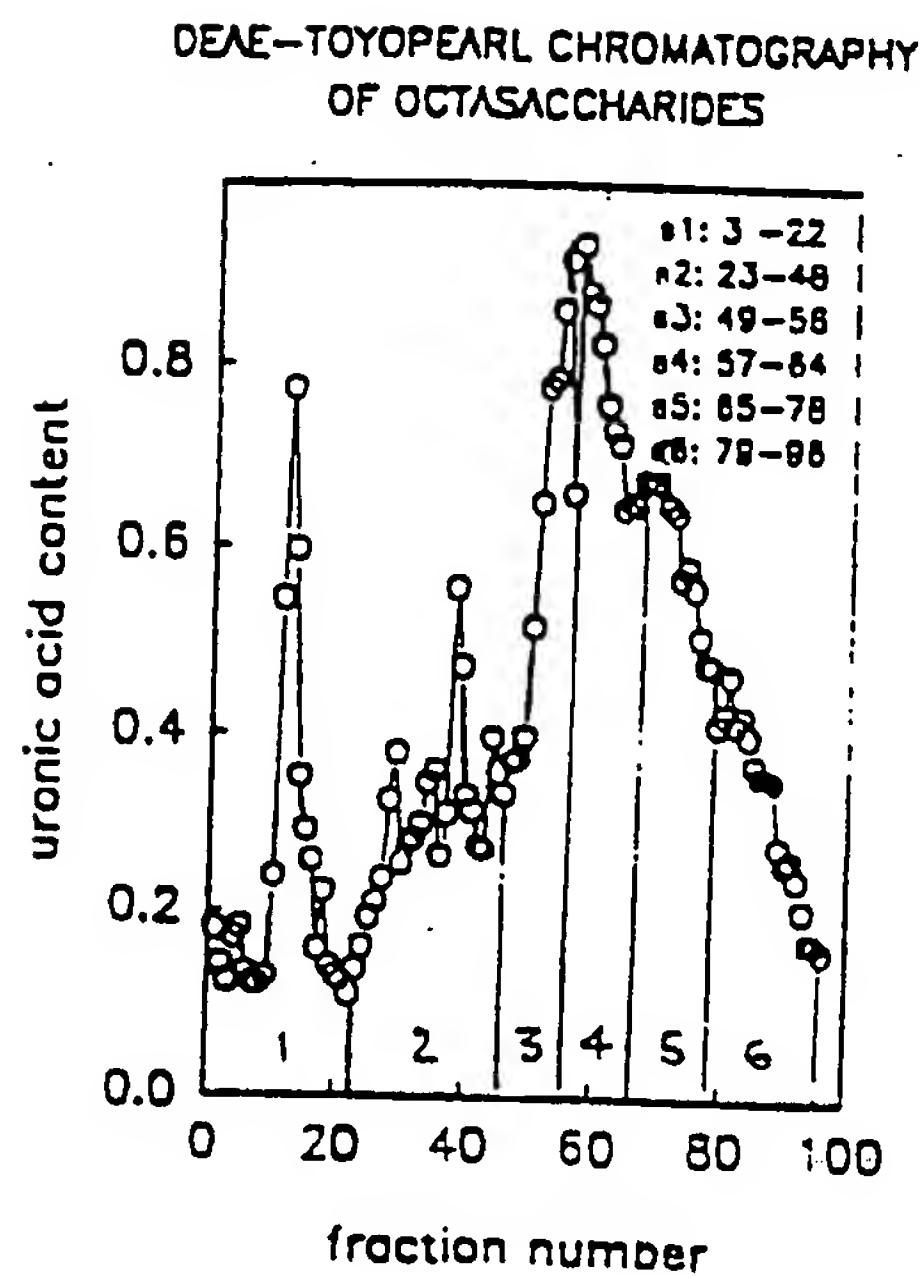
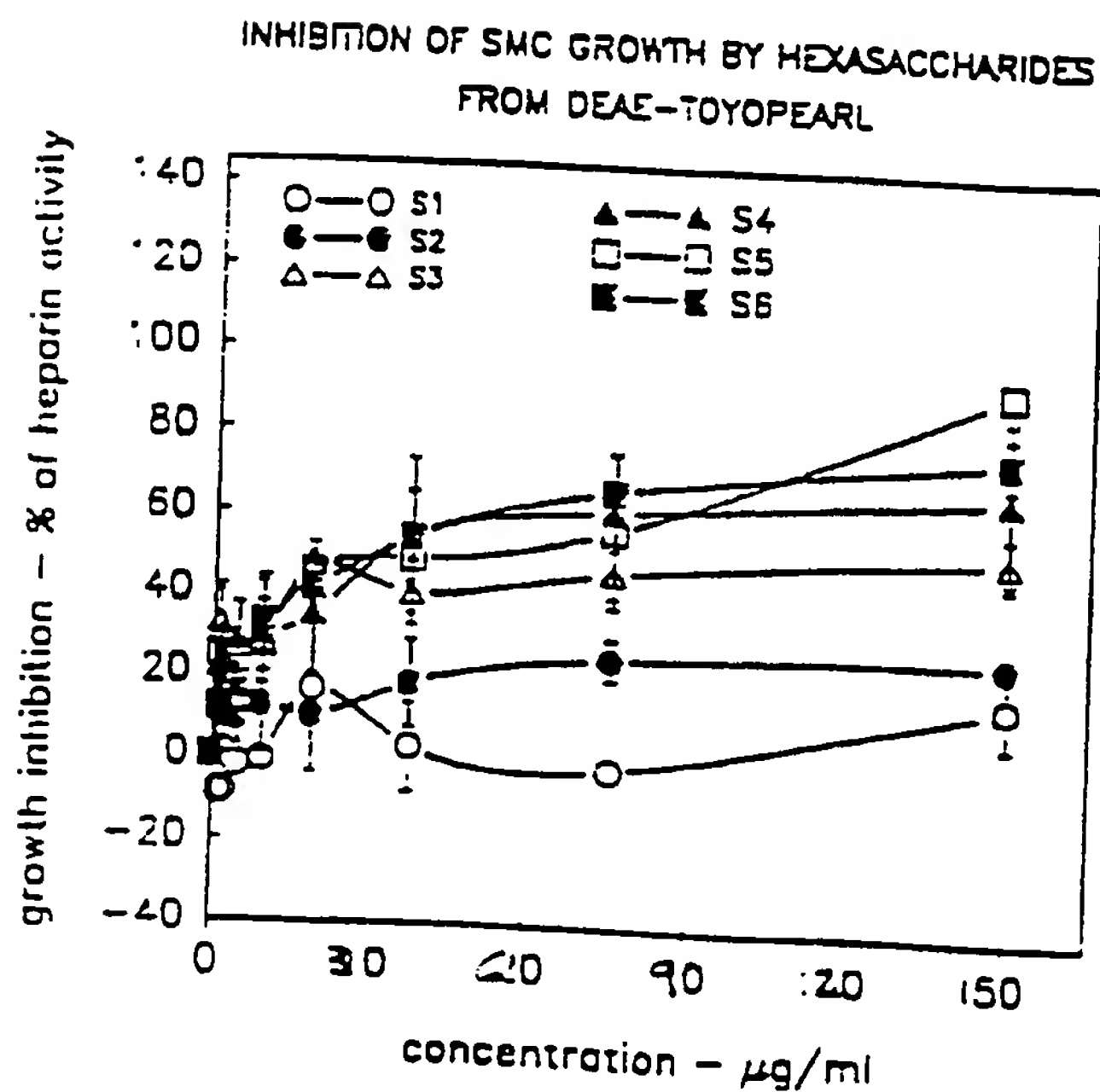
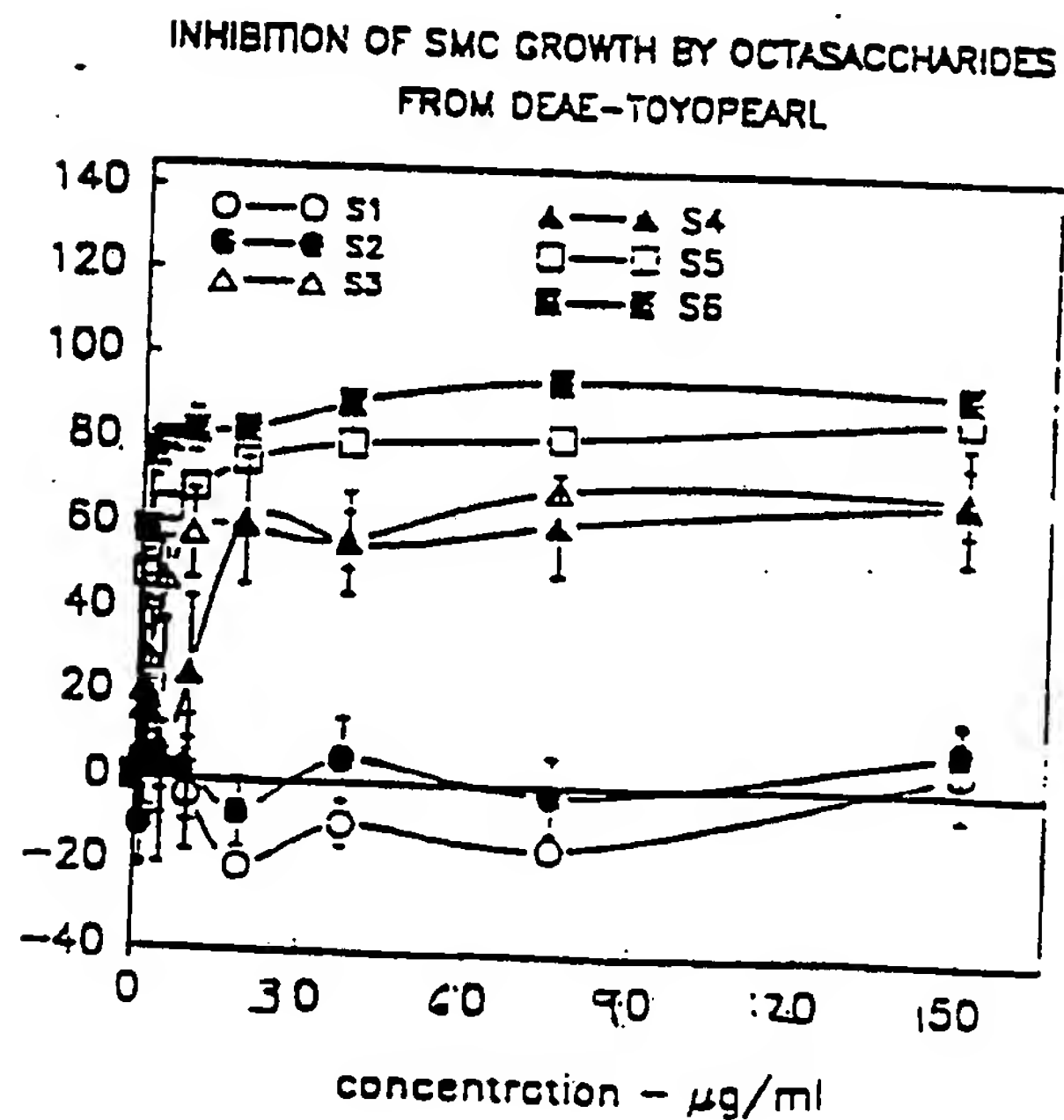


Fig. 3B



4A



4B

FIGURE 4

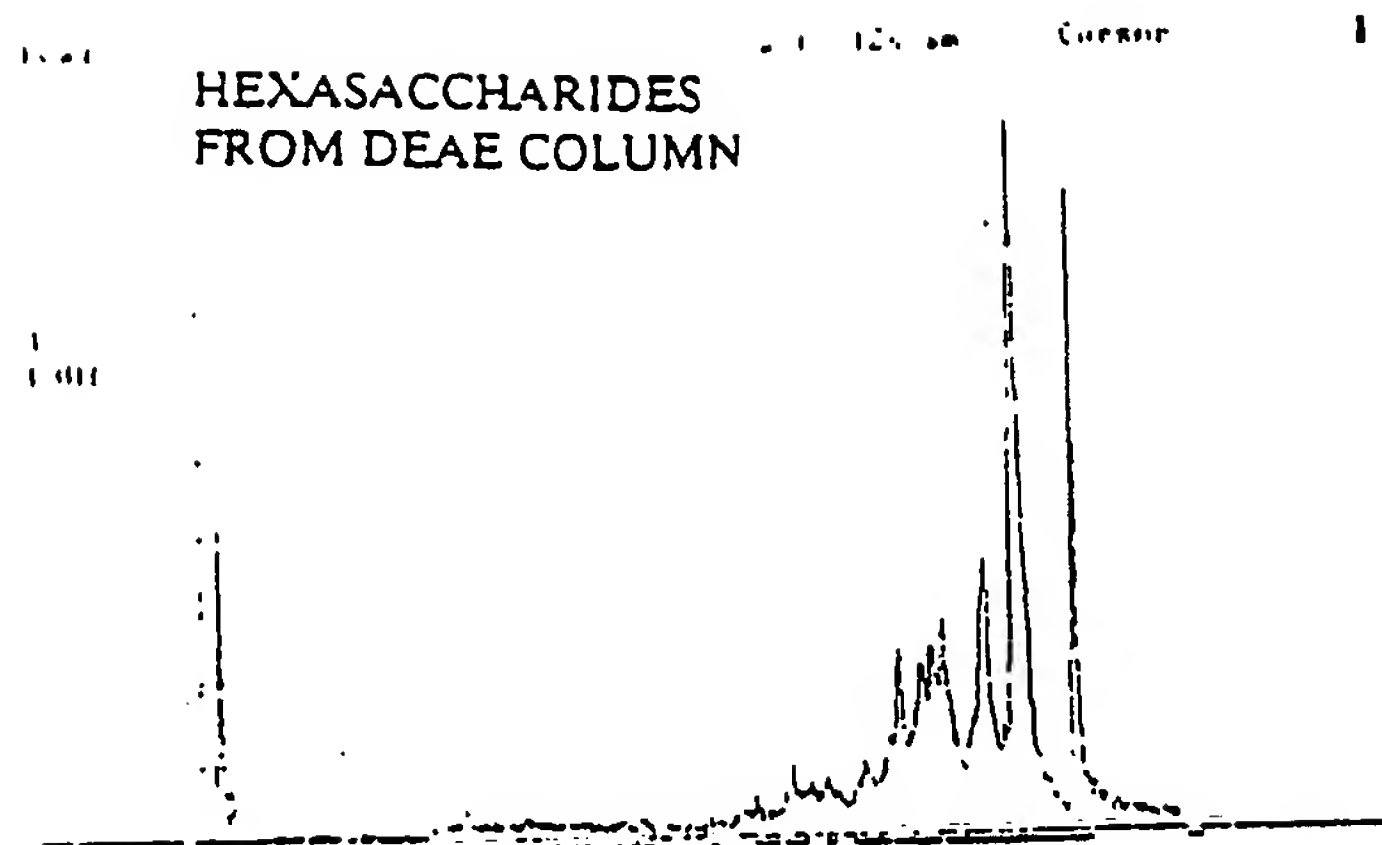


Fig. 5A

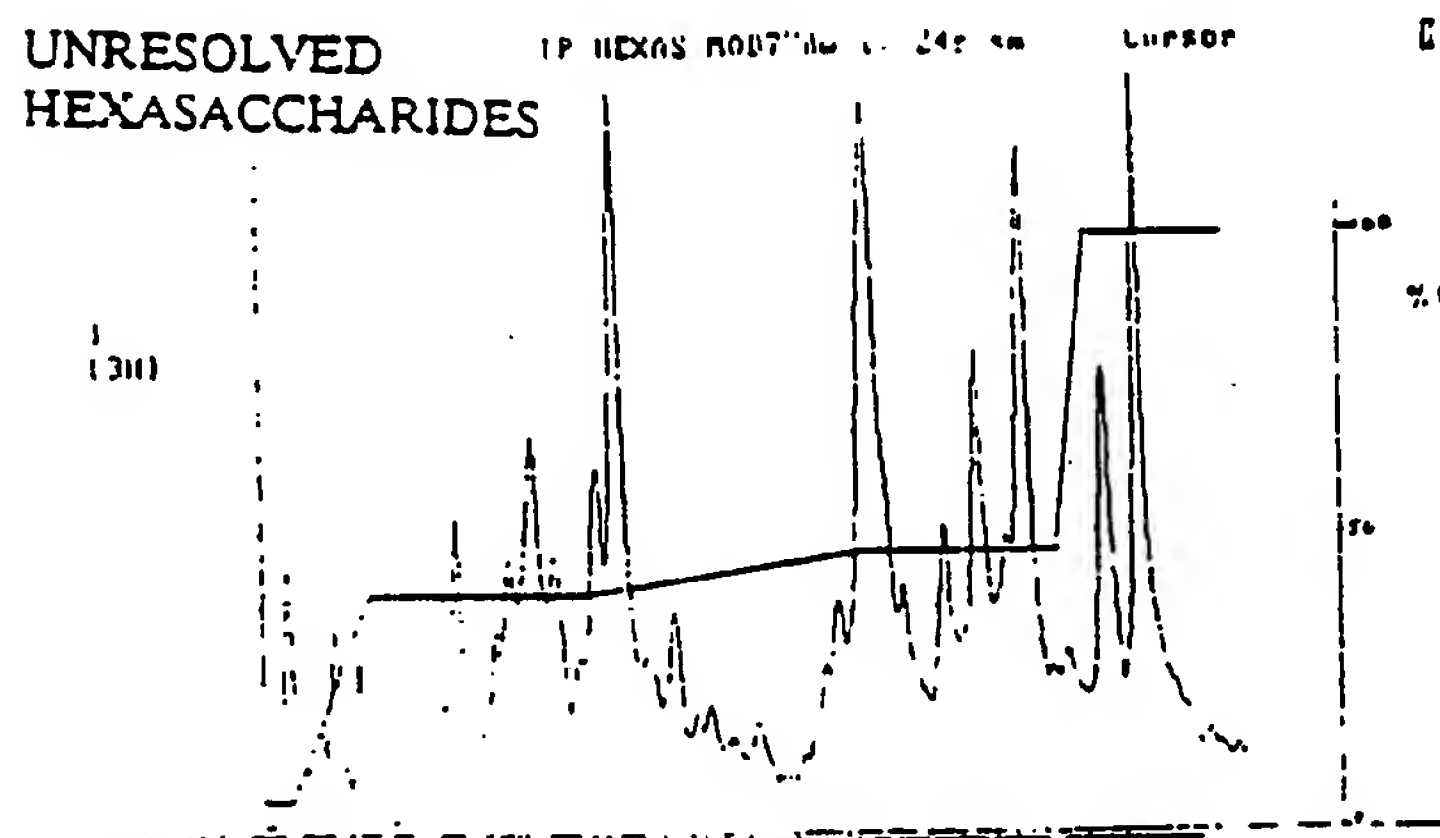


Fig. 5B

FIGURE 6A

n=2

	A	B	A'	B'	C'	D
1	X	X			X	X
2	X	X			X	
3	X	X				
4		X			X	X
5		X			X	
6	X	X				X
7					X	X
8	X				X	X
9	X				X	
10	X					X
11		X				X
12	X	X	X		X	X
13	X	X	X		X	
14	X	X	X			
15		X	X		X	X
16		X	X		X	
17	X	X	X			X
18			X		X	X
19	X		X		X	X
20	X		X		X	
21	X		X			X
22		X	X			X

FIGURE 6B

n=2

	A	B	A'	B'	C'	D
23	X	X		X	X	X
24	X	X		X	X	
25	X	X		X		
26		X		X	X	X
27		X		X	X	
28	X	X		X		X
29				X	X	X
30	X			X	X	X
31	X			X	X	
32	X			X		X
33		X		X		X
34	X	X	X	X	X	X
35	X	X	X	X	X	
36	X	X	X	X		
37		X	X	X	X	X
38		X	X	X	X	
39	X	X	X	X		X
40			X	X	X	X
41	X		X	X	X	X
42	X		X	X	X	
43	X		X	X		X
44		X	X	X		X

FIGURE 6C

n=2

	A	B	A'	B'	C'	D
45			X	X		
46	X		X	X		
47		X	X	X		
48			X	X	X	
49			X	X		X
50	X		X			
51		X	X			
52			X		X	
53			X			X
54	X			X		
55		X		X		
56				X	X	
57				X		X
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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US92/03092

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) :C08B 37/10; C07H 15/04, 17/04; A61K 31/715, 31/725

US CL :536/21, 17.2, 17.5, 17.9, 22; 514/42, 56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/21, 17.2, 17.5, 17.9, 22; 514/42, 56

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS (smooth muscle cell#)(p)(proliferation) = L1

L1(and)(inhibiting or inhibition or inhibit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Cell Biology, Volume 102, issued May 1986, "Structural Determinants of the Capacity of Heparin to Inhibit the Proliferation of Vascular Smooth Muscle Cells. II. Evidence for a Pentasaccharide Sequence that Contains a 3-O-Sulfate Group", pages 1979 - 1984, especially formula d of figure 1 on page 1980.	1-4 and 17
Y	Advances In Carbohydrate Chemistry and Biochemistry, Volume 43, issued 1985, Benito Casu, "Structure and Biological Activity of Heparin", pages 51-135, especially pages 68, 88 and 91-94.	5-16
Y	US, A, 4,401,662 (Lormeau et al) 30 August 1983.	1-17
Y	The Journal of Biological Chemistry, Volume 263, No. 11, issued 15 April 1988 (U.S.A.), G. Pejler et al, "Monoclonal Antibodies Specific for Oligosaccharides Prepared by Partial Nitrous Acid Deamination of Heparin", pages 5197-5201, especially Figure 6 of page 5200.	5-16

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 JULY 1992

Date of mailing of the international search report

28 JUL 1992

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